

PATENT APPLICATION

**PRODUCTION OF ATTENUATED PARAINFLUENZA VIRUS
VACCINES FROM CLONED NUCLEOTIDE SEQUENCES**

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08-26-98

PATENT

Attorney Docket No. 17634-000320
DHHS E-089-97/0

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Related Applications

c The present application is a continuation in part
c application of, and claims the benefit under Title 35 of U.S.
15 C C. 119(e) of Provisional Application No. 60/047,575, filed May 23, 1997,
^ and U.S. Provisional Application No. 60/059,385, filed
September 19, 1997, each of which is incorporated herein by
reference.

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Background of the Invention

Human parainfluenza viruses (HPIV), HPIV1, HPIV2, and HPIV3 are significant causes of bronchiolitis, croup and pneumonia in infants and children. Karron et al., J. Infect. Dis. 172: 1445-50 (1995); Collins et al. "Parainfluenza Viruses", p. 1205-1243. In B. N. Fields et al., eds., Fields Virology, 3rd ed, vol. 1. Lippincott-Raven Publ., Philadelphia (1996); Murphy et al., Virus Res. 11:1-15 (1988). Infections by these viruses result in substantial morbidity in children less than 3 years of age, and are responsible for approximately 20% of hospitalizations among young infants and children for respiratory tract infections.

Despite considerable efforts to develop effective vaccine therapies against HPIV, no approved vaccine agents have yet been achieved for any HPIV strain, nor for ameliorating HPIV related illnesses. To date, only two live attenuated PIV vaccine candidates have received particular attention. One of these candidates is a bovine PIV (BPIV) strain that is antigenically related to HPIV3, and which has been shown to protect animals against HPIV3. BPIV3 is attenuated, genetically stable and immunogenic in human infants and children (Karron et al., J. Inf. Dis. 171:1107-14

(1995a); Karron et al., J. Inf. Dis. 172:1445-1450, (1995b)). A second PIV3 vaccine candidate, JS cp45 is a cold-adapted mutant of the JS wildtype (wt) strain of HPIV3 (Karron et al., (1995b), supra; Belshe et al., J. Med. Virol. 10:235-42 (1982)). This live, attenuated, cold-passaged (cp) PIV3 vaccine candidate exhibits temperature-sensitive (ts), cold-adaptation (ca), and attenuation (att) phenotypes which are stable after viral replication *in vivo*. The cp45 virus is protective against human PIV3 challenge in experimental animals and is attenuated, genetically stable, and immunogenic in seronegative human infants and children (Hall et al., Virus Res. 22:173-184 (1992); Karron et al., (1995b), supra).

HPIV3 is a member of the Paramyxovirus genus of the Paramyxovirus family, order Mononegavirales. Its genome is a single strand of negative-sense RNA 15462 nucleotides (nt) in length (Galinski et al., Virology 165: 499-510, (1988); Stokes et al., Virus Res. 25:91-103 (1992)) and encodes at least eight proteins (Collins et al., supra, (1996); Galinski, supra, (1991); Spriggs and Collins, J. Gen. Virol. 67: 2705-2719, (1986)). Three of these proteins are associated with the RNA genome to form the nucleocapsid; namely the nucleocapsid protein N, phosphoprotein P, and large polymerase subunit L. Three additional proteins are associated with the envelope, namely the matrix protein M, taught to mediate viral attachment and release, the hemagglutinin-neuraminidase protein HN, and the fusion protein F. Two other proteins, HN and F, represent the neutralizing and protective antigens of PIVs (Collins et al. In Fields Virology, 3rd ed., 1:1205-43 (1996)). Significant sequence divergence in these two protective antigens among different PIVs is the basis for the type specificity of protective immunity against these pathogens (id.).

Another protein of PIV, the C protein, is encoded by an overlapping open reading frame (ORF) of the P protein mRNA (Spriggs and Collins, 1986), and the D protein is generated by RNA editing of the P cistron (Galinski et al. Virology 186:543-50 (1992)). The P mRNA also contains an internal ORF which has the potential to encode a cystein-rich domain called

V. The V ORF is also found in other paramyxoviruses and typically is accessed by RNA editing, but this is not the case with PIV. Presently, it is not known whether the PIV V ORF is expressed.

5 The viral genome of PIV also contains extragenic leader and trailer regions, possessing promoters required for viral replication and transcription. Thus, the PIV genetic map is represented as 3' leader-N-P/C/D-M-F-HN-L-trailer.
10 Transcription initiates at the 3' end and proceeds by a sequential stop-start mechanism that is guided by short conserved motifs found at the gene boundaries. The upstream end of each gene contains a gene-start (GS) signal, which directs initiation of its respective mRNA. The downstream terminus of each gene contains a gene-end (GE) motif which directs polyadenylation and termination.
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20 Identification of attenuating mutations in *cp45* and other PIV3 vaccine candidates is of interest for a variety of reasons. In particular, it will be useful to understand the genetic basis of attenuation and to characterize the molecular virology and pathogenesis of attenuated HPIV3 strains to provide clinically acceptable vaccines for use against these and other paramyxoviruses, especially HPIV1 and HPIV2 which together account for an additional 7% of pediatric hospital admissions. To achieve these and related goals, a method for
25 producing virus with a wt phenotype from cDNA is needed to determine which mutation(s) in the *cp45* virus specify the *ts*, *ca* and *att* phenotypes and which gene(s) of the BPIV3 specify the attenuation phenotype.

30 The complete nucleotide sequences of the prototype PIV3 strain, and of the JS wt HPIV3 and *cp45* strains have been determined (Stokes et al., supra., (1992); Stokes et al., *Virus Res.* 30: 43-52 (1993)). From these studies, the *cp45* strain was shown to possess at least seventeen nucleotide substitutions compared to the parental JS wt HPIV3 strain,
35 eight of which are correlated with changes to viral proteins. However, it has not been previously shown which of these identified mutations specify desired, e.g., *ts*, *ca*, and *att*, phenotypes. Recently, growth of *cp45* at nonpermissive

temperatures was reported to be complemented by coexpression of a cDNA clone of the L gene of the 47885 wt PIV3 strain (Ray et al., J. Virol. 70:580-584 (1996)), suggesting that the L gene may contain one or more mutations which contribute to the 5 ts phenotype of cp45. However, the results of this study are complicated by the fact that the 47885 strain is not isogenic with the JS parent of cp45 (for example, the two viruses are 4% different at the nucleotide level, and the L proteins differ at 41 amino acid positions (Stokes et al., supra, 10 (1992); published erratum appears in Virus Res. 27:96 (1993); Virus Res. 25:91-103. Also, this method of complementation does not provide a clear measurement of the relative contribution of the L gene mutation(s) to the overall ts phenotype of cp45.

15 Rescue and analysis of attenuating mutations in PIV3 and other RNA viruses require effective methods to manipulate cDNAs for the particular viruses of interest. Despite previous advancements identifying cDNAs for PIV, manipulation of the genomic RNA of this and other negative-sense RNA 20 viruses has proven difficult. One major obstacle in this regard is that the naked genomic RNA of these viruses is noninfectious.

Successful methods for direct genetic manipulation of non-segmented negative strand RNA viruses have only 25 recently begun to be developed (for reviews, see Conzelmann, J. Gen. Virol. 77:381-89 (1996); Palese et al., Proc. Natl. Acad. Sci. U.S.A. 93:11354-58, (1996)). Functional nucleocapsids have been successfully generated from the intracellular coexpression of separately transfected plasmids 30 bearing the T7 RNA polymerase promoter and encoding either genomic or antigenomic RNA and the N, P, and L proteins. The intracellular cDNA expression is driven by T7 RNA polymerase which is produced by co-infecting with a vaccinia recombinant virus. This approach was first used to determine the 35 transcription and replication requirements of cDNA-encoded minireplicons. Some success has been achieved in the application of these general methods to rescue infectious rabies virus, vesicular stomatitis virus (VSV), measles virus,

and Sendai virus from cDNA-encoded antigenomic RNA in the presence of the nucleocapsid N, phosphoprotein P, and large polymerase subunit L (Garcin et al., EMBO J. 14:6087-6094 (1995); Lawson et al., Proc. Natl. Acad. Sci. U.S.A.

5 92:4477-81 (1995); Radecke et al., EMBO J. 14:5773-5784 (1995); Schnell et al., EMBO J. 13:4195-203 (1994); Whelan et al., Proc. Natl. Acad. Sci. U.S.A. 92:8388-92 (1995)).

10 Respiratory syncytial virus (RSV) has also been recovered from cDNA encoded antigenome but this required the transfection of an additional plasmid encoding the M2 ORF 1 transcription elongation factor (Collins et al., 1995).

Rescue of infectious PIV virus and other Mononegavirales members is complicated by virtue of their non-segmented negative-strand RNA genome. The genomic ribonucleoprotein complexes (RNPs) of segmented genome viruses, such as influenza, are generally small in size and loosely structured, and can be assembled *in vitro* from RNA and required viral proteins. However, PIV and other Mononegavirales members feature much larger and more tightly structured RNPs, which tend to be refractory to functional association *in vitro*. Furthermore, the coding potential of HPIV3 P mRNA is complicated by cotranscriptional "RNA editing" (Galinski et al., Virology 186: 543-50 (1992)). The resultant shifts in reading frame can access internal ORFs which are expressed as chimeras fused to the N-terminal part of P. In addition, HPIV3 appears to differ from most other paramyxoviruses which express a chimeric V protein, as noted above. The corresponding set of proteins from HPIV3 editing has not yet been identified, and the internal V ORF of HPIV3 is separated from the editing site by numerous translational stop codons (Galinski et al. (1992, supra)). Yet another complicating factor is that editing by BPIV3 and HPIV3 produces a novel chimeric protein D, in which the upstream half of P is fused to a domain encoded by a second internal ORF (Pelet et al., EMBO J. 10: 443-448 (1991); Galinski et al., supra, (1992)). The D protein does not have a counterpart in other paramyxoviruses.

In view of the foregoing, an urgent need exists in the art for tools and methods to engineer safe and effective vaccines to alleviate the serious health problems attributable to PIV, particularly illnesses among infants and children attributable to HPIV3. Quite surprisingly, the present invention satisfies these and other related needs.

Summary Of The Invention

The present invention provides novel tools and methods for introducing defined, predetermined structural and phenotypic changes into infectious PIV. In one embodiment of the invention, an isolated polynucleotide molecule is provided which comprises an operably linked transcriptional promoter, a polynucleotide sequence encoding a PIV genome or antigenome, and a transcriptional terminator.

The PIV genome or antigenome can be a human or nonhuman PIV sequence, or a recombinantly modified version thereof. In one embodiment, the polynucleotide sequence encodes a chimeric genome or antigenome comprising a human PIV sequence recombinantly joined with a nonhuman PIV sequence, such as a gene or gene segment from bovine PIV (BPIV). In additional examples, the polynucleotide encodes a chimera of sequences from a nonhuman PIV and at least one other PIV of human or nonhuman origin.

In other embodiments, the invention provides an isolated infectious PIV particle comprising a recombinant PIV (rPIV) genome or antigenome. The isolated infectious PIV particle can be a viral or subviral particle. As used herein, subviral particle refers to any infectious PIV particle which lacks a structural element, e.g., a gene segment, gene, protein, or protein functional domain, which is present in a complete virus (e.g., an assembled virion including a complete genome or antigenome, nucleocapsid and envelope). Thus, one example of a subviral particle of the invention is an infectious nucleocapsid containing a genome or antigenome, and the products of N, P, and L genes. Other subviral particles are produced by partial or complete deletions or substitutions

of non-essential genes and/or their products (eg., F, HN, M, or C), among other non-essential structural elements.

The isolated infectious PIV particle is preferably a human PIV, more preferably human PIV3 (HPIV3). The invention also provides isolated, infectious particles from bovine or murine PIV (BPIV or MPIV), as well as particles comprising chimeric sequences from two or more different PIV genomes, for example particles incorporating polynucleotide sequences from HPIV3 and HPIV1, from HPIV3 and HPIV2 sequences, or comprised of HPIV3 and BPIV sequences.

In related aspects of the invention, isolated, infectious PIV particles are provided which incorporate nucleotide sequences from HPIV3 joined to at least one sequence from a heterologous PIV, such as HPIV1, HPIV2, BPIV or MPIV. For example, entire genes of HPIV3 may be replaced by counterpart genes from other forms of PIV, such as the HN and/or F glycoprotein genes of PIV1 or PIV2. Alternatively, a selected gene segment, for example a cytoplasmic tail, transmembrane domain or ectodomain of HN or F of HPIV1 or HPIV2, can be substituted for a corresponding gene segment in a counterpart HPIV3 gene to yield constructs encoding chimeric proteins, e.g. fusion proteins having a cytoplasmic tail and/or transmembrane domain of PIV3 fused to an ectodomain of PIV1 or PIV2. Alternatively, genes or gene segments from one PIV can be added (i.e., without substitution) within a heterologous PIV background to create novel immunogenic properties within the resultant clone.

Other modifications can be produced by introducing into a PIV genome or antigenome a nucleotide insertion, rearrangement, deletion or substitution selected to encode a desired phenotypic alteration, such as one that results in attenuation, temperature-sensitivity, cold-adaptation, small plaque size, host range restriction, improved growth in vitro or a change in an immunogenic epitope of PIV. In one aspect of the invention, mutations occurring in biologically derived, attenuated PIV are identified and introduced individually or in combination into a full-length PIV clone. Typically these mutations are single amino acid changes

displayed by biologically derived mutant viruses over a wild-type PIV, for example changes exhibited by PIV mutants having *ts*, *ca* or *att* phenotypes. These changes from biologically derived mutant PIV are incorporated into a recombinant PIV clone to specify desired characteristics in the resultant virus. Exemplary mutations include amino acid changes which specify an attenuated phenotype in the HPIV3 strain JS *cp45*. Among these exemplary mutations are mutations occurring within the PIV polymerase gene L specifying *ts*, *ca* or *att* phenotypes, for example amino acid substitutions occurring at Tyr_{942} , Leu_{992} , and/or Thr_{1558} of the JS wild type PIV strain. In more detailed aspects, attenuated PIV recombinants are described wherein Tyr_{942} is replaced by His, Leu_{992} is replaced by Phe, and/or Thr_{1558} is replaced by Ile.

Also provided within the invention are recombinant PIV having multiple, phenotype-specifying mutations introduced in selected combinations into the genome or antigenome of an infectious clone to yield desired characteristics including attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc. For example, PIV clones are provided which incorporate at least two separate mutations adopted from a biologically derived PIV mutant, e.g., two *ts* mutations from HPIV3 JS *cp45*. Multiply attenuated viruses are thus obtained by selecting mutations from a "menu" of identified lesions and introducing these mutations in various combinations to calibrate a vaccine virus to selected levels of attenuation, immunogenicity and stability.

In additional embodiments, the invention provides for supplementation of one or more mutations adopted from biologically derived PIV, e.g., *ts*, *ca* or *att* mutations, with additional types of mutations involving the same or different genes. Target genes for mutation in this context include the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F and the C, D and V ORF products. In preferred aspects, attenuating mutations adopted from biologically derived PIV are incorporated within a chimeric

PIV recombinant, e.g., a PIV recombinant having nucleotide sequences from both HPIV3 and HPIV1, or from both HPIV and BPIV viruses.

In other embodiments, the invention provides methods for producing an infectious PIV particle, e.g., a viral or subviral particle, from one or more isolated polynucleotide molecules encoding a PIV genome or antigenome (see also copending United States provisional patent application No. 60/047575, filed May 23 1997, incorporated herein by reference in its entirety). To produce an infectious PIV particle according to these methods, an expression vector comprising an isolated polynucleotide molecule encoding a PIV genome or antigenome is coexpressed in a cell or cell-free system with an expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins of a PIV, whereby an infectious PIV particle is produced.

The PIV genome or antigenome and the N, P, and L proteins may be coexpressed by a single expression vector, or by separate expression vectors. In alternate embodiments, the N, P, and L proteins are each encoded on separate expression vectors.

Within the aforementioned methods, the polynucleotide molecule encoding the PIV genome or antigenome may correspond to a genomic or antigenomic sequence of human, bovine or murine PIV. Alternatively, the PIV encoding polynucleotide may be a chimera of a human PIV genomic or antigenomic sequence and at least one non-human PIV genomic or antigenomic sequence. In additional methods for producing infectious PIV, the polynucleotide encoding the PIV genome or antigenome is a chimera of two or more human PIV genomes, for example a polynucleotide containing sequences from HPIV3 joined to sequences from one or more related forms of human PIV, such as human PIV1 or human PIV2. Individual genes of human PIV3 may be substituted by counterpart genes from heterologous PIV, for example the HN and F glycoprotein genes of PIV1 or PIV2, to yield a modified genome or antigenome encoding a chimeric PIV. Alternatively, a selected, heterologous gene segment, such as a cytoplasmic tail,

transmembrane domain or ectodomain of HN or F of HPIV1 or HPIV2, can be substituted for a counterpart gene segment in a different PIV type or different gene, e.g., HN or F of HPIV3, to yield constructs encoding chimeric proteins, e.g. fusion 5 proteins having a cytoplasmic tail and/or transmembrane domain of PIV3 fused to an ectodomain of PIV1 or PIV2.

In yet additional methods for producing infectious PIV, the PIV genome or antigenome is modified to yield a chimera of a human PIV genomic or antigenomic sequence and at 10 least one non-human PIV sequence, for example a polynucleotide containing sequences from both human and bovine PIV.

In other methods for producing infectious PIV, the PIV genome or antigenome is modified by a nucleotide insertion, rearrangement, deletion or substitution selected to encode a desirable phenotypic alteration, such as one that results in attenuation, temperature-sensitivity, cold-adaptation, small plaque size, host range restriction, or a change in an immunogenic epitope of PIV. Alternatively, the polynucleotide molecule encoding the PIV genome or antigenome can be modified to encode non-PIV molecules, e.g., a cytokine, a T-helper epitope, a restriction site marker, or a protein of a different microbial pathogen (e.g., virus, bacterium or fungus) capable of eliciting a protective immune response in the intended host. In one embodiment, the PIV genome or 20 antigenome is modified to encode protein from a human RSV or from measles virus.

In other embodiments of the invention a cell or cell-free expression system (e.g., a cell-free lysate) is provided which incorporates an expression vector comprising an isolated polynucleotide molecule encoding a PIV genome or antigenome, and an expression vector comprising one or more isolated polynucleotide molecules encoding N, P, and L proteins of a PIV. Upon expression, the genome or antigenome and N, P, and L proteins combine to produce an infectious PIV 30 particle, such as a viral or subviral particle. The isolated polynucleotide molecules encoding the PIV genome or antigenome and the one or more isolated polynucleotide molecules encoding N, P, and L proteins of PIV can be expressed by a single 35

vector, or the genome and one or more of the N, P, and L proteins can be incorporated into two or more separate vectors.

5 Brief Description of the Drawings

Figure 1 illustrates construction of the plasmid p218(131), encoding a complete copy of HPIV3 genomic RNA. The diagram of p218(A*A'CC'D*E) (top left) illustrates the location of the T7 terminator and delta ribozyme adjacent to nucleotide 1 of the HPIV3 genome. The top right diagram of p(E*FF'GHIJKL*) illustrates the locations of the 7 sequencing markers (asterisk), see Fig. 2 below, and the T7 promoter adjacent to nucleotide 15462 of HPIV3. The 15 overlapping clones used to construct the full length genomic cDNA are indicated by letters on the outside of the two plasmids. The unique restriction sites *Ngo*MI and *Xho*I were used to clone p218(131) which encodes the complete negative-sense genomic RNA of HPIV3 illustrated at the bottom. The location of each sequencing marker in p218(131) differentiating recombinant JS from JS wt is indicated by an asterisk.

Figure 2 depicts sequence markers in cDNA encoded genomic RNA of the HN (1) and L(2) genes of HPIV3. Sequences are negative sense, mutations are boxed.

1) A non-coding nt substitution at 7903 removes a *Hga* I site from JS wt. Nucleotide substitutions in JS cDNA at 7913 and 7915 create a *Sca* I site and change amino acid 370 from proline to threonine, ablating the antibody epitope recognized by mAb 170/7 and 423/6. Also, the HN gene was found to contain an additional point mutation at nt 7593 which had not been recognized previously. This results in a threonine to isoleucine change at amino acid position 263 in the HN protein.

2) The three incidental non-coding mutations in the L gene of JS cDNA that occurred during plasmid construction or assembly are boxed.

Figure 3 includes a diagram (not to scale) of the PIV3-CAT cDNA (top, drawn as a circular plasmid) and the

encoded minigenome RNA (bottom, drawn as a single strand of negative-sense RNA, 3' to 5').

Plasmid: The T7 promoter is shown as a black arrow pointing in the direction of transcription. Positions are shown for the delta ribozyme, the T7 transcriptional terminator, the inserted vaccinia virus transcriptional terminators (T₇CT and T₅AT), restriction enzyme sites, and the coding sequence for the various domains of the encoded minigenome.

Minigenome RNA: The 5' (right hand) end of the minigenome is defined by the promoter for T7 RNA polymerase, which contributes an extension of two nonviral G residues. These are not included in length calculations. The 3' end is defined by the ribozyme and is free of any heterologous nucleotides. PIV3-specific regions are shown as open boxes. The CAT ORF is shaded and the negative-sense complements of the translational initiation (UAC) and termination (AUU) codons are shown. Nucleotide positions are indicated according to the shortest, 898-nucleotide minigenome.

Nucleotide lengths of specific regions are given in parentheses. The sequence of positions 90 to 124 (3' to 5', negative-sense) is shown at the bottom; PIV3-specific sequence is underlined, the complement of a vaccinia virus transcriptional terminator is overlined, the complement of the XbaI site is italicized, and the complement of the translational start codon for the CAT ORF is in bold. The site where 0 to 6 G residues were inserted, resulting in PIV3-CAT 0 to +6, is indicated. Abbreviations: GS, transcriptional gene-start motif; GE, transcriptional gene-end termination/polyadenylation motif; NT, nontranslated gene sequence.

Figure 4 depicts expression of CAT enzyme by minigenomes PIV3-CAT 0 to +6. Cells were transfected with plasmids encoding the indicated PIV3-CAT minigenome and the N, P and L proteins, and were infected simultaneously with vaccinia virus recombinant vTF7-3 expressing T7 RNA polymerase. Lysates were prepared 48 h post-infection, and samples representing 3000 cells were analyzed for CAT

activity. Activities are expressed relative to the +2 minigenome, which is a multiple of six, as 100. Samples in which the L plasmid was omitted did not have CAT activity detectable under these conditions.

Figures 5A and 5B depict synthesis of mini-antigenomic RNA by PIV3-CAT minigenomes 0 to +6. Cells were transfected and infected as described for Fig. 4, except for controls in which N plasmid (lane 1) or L plasmid (lane 2) was omitted. The PIV3-CAT minigenome plasmid which was transfected in each case is indicated. Cell lysates were prepared at 48 h post-transfection: one aliquot was treated with micrococcal nuclease (treated) and the second was mock-treated (untreated), followed by processing for RNA purification. The RNAs were analyzed by Northern blot hybridization with a negative-sense CAT riboprobe. As a marker and control for nuclease treatment, the RSV-CAT C2 minigenome (Grosfeld et al., *J. Virol.* 69:5677-5686 (1995), incorporated herein by reference in its entirety) was complemented by the RSV N, P and L proteins and was processed in parallel (Lane 10). Autoradiograms of the hybridized blots are shown in Fig. 5A. Fig. 5B shows phosphorimager analysis in which the amount of hybridization relative to the +2 minigenome was calculated separately for the treated and untreated samples.

Figures 6A and 6B depict synthesis of polyadenylated mRNA by PIV3-CAT minigenomes 0 to +6. Using aliquots of cells from the same experiment as shown in Figs. 5A and 5B, total intracellular RNA was harvested at 48 h post-transfection and fractionated by oligo(dT) chromatography into bound and unbound fractions, which were analyzed by Northern blot hybridization with a negative-sense CAT riboprobe. Fig. 6A shows autoradiograms of the hybridized blots. Fig. 6B shows the phosphorimager analysis in which the amount of hybridization was normalized relative to the bound RNA fraction of the +2 minigenome, this was calculated separately for the bound and unbound fractions.

Figures 7A and 7B depict accumulation of intracellular minigenome in response to plasmid encoding

PIV3-CAT minigenomes 0 to +6. This is a continuation of the experiment shown in Figs. 5A and 5B. Thus, cells which had been transfected with plasmids encoding a minigenome and the N, P and L proteins and infected with vTF7-3 were harvested 48 h post-transfection and cell lysates were prepared. One aliquot of lysate was treated with micrococcal nuclease (treated) and the other was mock-treated (untreated), followed by processing for RNA purification. The RNAs were analyzed by Northern blot hybridization with a positive-sense CAT riboprobe, whereas in Fig. 5A the riboprobe was negative-sense. Fig. 7A shows autoradiograms of the hybridized blots. Fig. 7B shows phosphorimager analysis in which the amount of hybridization relative to the +2 minigenome was calculated separately for the treated and untreated samples.

Figures 8-10 illustrate construction of p3/7(131)2G. p3/7(131)2G encodes a complete positive-sense HPIV3 antigenome and contains two G residues between the T7 promoter and nucleotide 1 of the antigenome. The construction involved modification separately of the left and right halves of cDNA 218(131) encoding the negative-sense genome. Using the same strategy, a second cDNA, p3/7(131) was constructed which is identical to p3/7(131)2G except that the two G residues were omitted.

Figure 8 depicts construction of p(Left+2G), performed by replacing the ribozyme and T7 transcription terminator of p218(A*A'CC'D*E) with a T7 promoter including two G residues. p218 (A*A'CC'D*E) was used as the template in a PCR that amplified the left-hand 1224 nucleotides of the HPIV3 genome (black rectangle labeled "PIV3seq.") using a left hand PCR primer that introduced the T7 promoter. This PCR fragment was cloned into the MluI-PmlI window of p218 (A*A'CC'D*E), resulting in p(Left+2G).

Figure 9 depicts construction of p(Right+), performed by replacing the T7 promoter of p(E*FF'GHIHKL*) with a ribozyme and T7 terminator placed adjacent to PIV3 nucleotide 15642 in the positive-sense (antigenome) strand. p(E*FF'GHIHKL*) was used as a template in a PCR that amplified

the right-hand 649 nucleotides of the HPIV3 genome (black rectangle labeled "PIV3 seq.") using a mutagenic oligonucleotide that added part of the delta ribozyme (including a naturally-occurring RsrII site). This PCR product was cloned into the RsrII-BssHII window of p3/7 to yield pPIV3-3/7, thus reconstructing a complete ribozyme flanked by the T7 terminator. The SwaI-NgoMI fragment of pPIV3-3/7 was cloned into the SwaI-NgoMI windows of p(E*FF'GHIHKL*), resulting in p(Right+). The locations of the seven sequence markers are indicated with asterisks.

Figure 10 depicts construction of p3/7(131)2G. The NgoMI-XhoI fragment of p(Right+) was cloned into the NgoMI-XhoI window of p(Left+2G), resulting in p3/7(131)2G. The positions of HPIV3 genes are indicated (not to scale). The locations of sequence markers are indicated with asterisks.

Figure 11 shows sequence confirmation of a negative sense recombinant PIV3. A 1379 bp fragment (nucleotides 7334-8713) spanning the mutations at 7903, 7913, and 7915 was generated by RT-PCR of RNA from infected cells and then analyzed by cycle-sequencing. The mutations differentiating recombinant PIV from JS wt are indicated by arrows. The complete sequence from nt 7897 to 7922 is shown in the margin next to each gel, with the three nucleotide differences indicated in bold.

Figure 12 provides a map of plasmid pTM(L) 942/992/1558, which contains the PIV3 L cDNA with amino acid substitutions at positions 942, 992, and 1558 in the L protein sequence. The relative position of each of coding change is indicated, together with the aa difference and the naturally-occurring restriction site which was deliberately ablated as a marker. Restriction sites used for cloning (*Sph*I, *Pin*A_I, *Bam*H_I and *Nhe*I) are indicated. The arrow shows the direction of the L protein coding sequence and is numbered according to amino acid position.

Figure 13 is a schematic representation of recombinant PIV3 viruses bearing representative mutations within the invention.

Figure 14 illustrates construction of cDNA encoding the chimeric PIV3-PIV1 antigenome in which the PIV3 HN and F ORFs were replaced by those of PIV1. First (starting from the bottom left) the PIV3 F and HN genes were subcloned (as *BspE*-I 5 *I-Xho* I and *EcoR* I-*Spe* I fragments) from the full-length PIV3 cDNA clone p3/7(131)2G (Panel A) to generate pLit.PIV3.F3a and pLit.PIV3.HN4 (Panel B), respectively. PCR mutagenesis (Panel C) was performed to delete the complete coding regions of the PIV3 F and HN genes and to introduce new restriction sites 10 (boxed). PIV1 F and HN cDNAs were prepared from infected-cell RNA by RT-PCR and cloned into pLITMUS28 (Panel D). These clones, pLit.1Fwt and pLit.1HNwt, were used as templates for modification of the PIV1 F and HN genes at their start and stop codons to introduce new restriction sites compatible with those introduced in the PIV3 deletions (Panel E). Chimeric F 15 and HN genes were constructed by importing the PIV1 coding regions into the PIV3 deletions to generate pLit.PIV3-1.Fhc and pLit.PIV3-1.HNhc (Panel F). The chimeric F and HN were assembled together to generate pSE.PIV3-1.hc (Panel G). The F 20 and HN chimeric segment was introduced into full-length PIV3 clone p3/7(131)2G by replacing its *BspE* I-*Sph* I fragment to generate pFLC.2G+.hc (Panel H). The small boxes between genes represent the gene end, intergenic, and gene start regions, and the lines represent the non-coding regions. Shaded 25 portions are from PIV1, open boxes are from PIV3. The black arrows depict the T7 promoter, while black boxes depict the hepatitis delta virus (HDV) ribozyme.

Figure 15A is a diagram of the chimeric HN and F genes of the chimeric rPIV3-1 (middle) in parallel with that 30 of rPIV3/JS (top; alternatively referred to herein as rJS and JS wt rPIV3) and PIV1/Wash64 (bottom). The four junction regions containing the sequence transitions from PIV3 to PIV1 are boxed and numbered I to IV. Each small box between genes represents the gene end, intergenic, and gene start region, 35 and the lines represent the non-coding regions. RT-PCR primers, specific to PIV3 M and L genes (primer pair A at top) or specific to PIV1 M and HN genes (primer pair B at bottom) used in Figure 14B, are depicted as arrows.

Figure 15B shows an evaluation of RT-PCR products generated using virion RNA (vRNA) as template and the PIV3- or PIV1-specific primer pairs described in Figure 14A. The reverse transcription step was omitted in a duplicate of each template, as indicated, to confirm that the PCR product was derived from RNA. For rPIV3-1, the PIV3-specific primer pair A gave rise to the expected 4.6 kb product, whereas primer pair B, specific to PIV-1 sequences not present in rPIV3-1, did not yield product. Positive controls using rPIV3/JS (labeled as rPIV3) or PIV1/Wash64 (labeled as PIV1) vRNA are shown in parallel.

Figure 16 represents sequences of PIV3-PIV1 junctions in the RT-PCR products of rPIV3-1 shown in Figure 14B were determined. The sequence for each of the four junction regions (Regions I-IV) is presented and aligned with the corresponding regions of rPIV3/JS (top line) and PIV1/Wash64 (bottom line), which were sequenced in parallel from RT-PCR products. Vertical bars indicate sequence identity, and the boxed regions indicate introduced mutations and restriction sites. The Gln to Glu codon change in the chimeric F gene is indicated by shaded box. Start and stop codons are underlined.

Figure 16 shows sequencing gels for region III (left) and IV (right) of RT-PCR products of rPIV3-1 compared with rPIV3 (left) or PIV1 (right). Start and stop codons are marked by a box, restriction sites are marked by arrows.

Figure 18 depicts multicycle growth of parental and chimeric PIVs in tissue culture. LLC-MK2 cell monolayers were inoculated with virus at an MOI of 0.01, and virus-infected cells were incubated at 32°C in the presence of trypsin. Tissue culture supernatants were harvested at 24 hour intervals, frozen, and analyzed in the same TCID₅₀ assay using hemadsorption to identify virus-infected cultures. Each point represents the mean titer of three separate cultures, with S.E. Indicated. The dotted horizontal line indicates the lower limit of viral detection.

Figure 19 illustrates introduction of the three L gene mutations of cp45 into pFLC.2G+.hc, the antigenomic cDNA

clone of the chimeric virus rPIV3-1. pTM(L) 942/992/1558 (A) is a plasmid clone of the L gene that carries the three mutations found in cp45. The mutation at amino acid position 942 in the PIV3 L protein is a tyr (wt) to his (cp45) 5 substitution and a nearby Eae I site was ablated to mark this site. Similarly, the 992 mutation is a leu to phe change with a Bsr I site ablated and the 1558 mutation is a thr to ile change with an Ava II site ablated. The 2.9 kb SphI-NheI fragment present in pTM(L) 942/992/1558 was introduced into 10 pFLC.2G+hc (B), the plasmid carrying the full length cDNA clone of rPIV3-1, to give pFLC+hc.cp45L (C). For the constructs in (B) and (C), the black boxes indicate the location of the hepatitis D virus ribozyme and the T7 15 terminator, the shaded regions are the PIV1 HN and F ORFs, and the open boxes represent sequences derived from PIV3. pFLC.2G+.hc.cp45L was used in the transfection to yield the attenuated chimeric recombinant virus designated rPIV3-1.cp45L.

20 Description of the Specific Embodiments

The present invention provides compositions and methods for producing and modifying infectious PIV from isolated polynucleotide molecules, preferably cDNA. Infectious PIV particles are produced by a recombinant 25 coexpression system that permits introduction of defined changes into infectious PIV. These modifications are useful in a wide variety of applications, including the development of live attenuated vaccine strains bearing predetermined, defined attenuating mutations.

Infectious PIV of the invention are produced by intracellular or cell-free coexpression of one or more isolated polynucleotide molecules that encode a PIV genome or antigenome RNA, together with one or more polynucleotides encoding viral proteins necessary to generate a transcribing, 35 replicating nucleocapsid. Among the viral proteins useful for coexpression to yield infectious PIV are the major nucleocapsid protein (N) protein, nucleocapsid phosphoprotein (P), large (L) polymerase protein, fusion protein (F),

hemagglutinin-neuraminidase glycoprotein (HN), and matrix (M) protein. Also useful in this context are products of the C, D and V ORFs of PIV.

CDNAs encoding a PIV genome or antigenome are constructed for intracellular or *in vitro* coexpression with the necessary viral proteins to form infectious PIV. By "PIV antigenome" is meant an isolated positive-sense polynucleotide molecule which serves as a template for synthesis of progeny PIV genome. Preferably a cDNA is constructed which is a positive-sense version of the PIV genome corresponding to the replicative intermediate RNA, or antigenome, so as to minimize the possibility of hybridizing with positive-sense transcripts of complementing sequences encoding proteins necessary to generate a transcribing, replicating nucleocapsid.

In some embodiments of the invention the genome or antigenome of a recombinant PIV (rPIV) need only contain those genes or portions thereof necessary to render the viral or subviral particles encoded thereby infectious. Further, the genes or portions thereof may be provided by more than one polynucleotide molecule, i.e., a gene may be provided by complementation or the like from a separate nucleotide molecule. In other embodiments, the PIV genome or antigenome encodes all functions necessary for viral growth, replication, and infection without the participation of a helper virus or viral function provided by a plasmid or helper cell line.

By "recombinant PIV" is meant a PIV or PIV-like viral or subviral particle derived directly or indirectly from a recombinant expression system or propagated from virus or subviral particles produced therefrom. The recombinant expression system will employ a recombinant expression vector which comprises an operably linked transcriptional unit comprising an assembly of at least a genetic element or elements having a regulatory role in PIV gene expression, for example, a promoter, a structural or coding sequence which is transcribed into PIV RNA, and appropriate transcription initiation and termination sequences.

To produce infectious PIV from a cDNA-expressed PIV genome or antigenome, the genome or antigenome is coexpressed

with those PIV N, P and L proteins necessary to (i) produce a nucleocapsid capable of RNA replication, and (ii) render progeny nucleocapsids competent for both RNA replication and transcription. Transcription by the genome nucleocapsid provides the other PIV proteins and initiates a productive infection. Alternatively, additional PIV proteins needed for a productive infection can be supplied by coexpression.

Synthesis of PIV antigenome or genome together with the above-mentioned viral proteins can also be achieved *in vitro* (cell-free), e.g., using a combined transcription-translation reaction, followed by transfection into cells. Alternatively, antigenome or genome RNA can be synthesized *in vitro* and transfected into cells expressing PIV proteins.

In certain embodiments of the invention, complementing sequences encoding proteins necessary to generate a transcribing, replicating PIV nucleocapsid are provided by one or more helper viruses. Such helper viruses can be wild type or mutant. Preferably, the helper virus can be distinguished phenotypically from the virus encoded by the PIV cDNA. For example, it is desirable to provide monoclonal antibodies which react immunologically with the helper virus but not the virus encoded by the PIV cDNA. Such antibodies can be neutralizing antibodies. In some embodiments, the antibodies can be used in affinity chromatography to separate the helper virus from the recombinant virus. To aid the procurement of such antibodies, mutations can be introduced into the PIV cDNA to provide antigenic diversity from the helper virus, such as in the HN or F glycoprotein genes.

In alternate embodiments of the invention, the N, P, L and other desired PIV proteins are encoded by one or more non-viral expression vectors, which can be the same or separate from that which encodes the genome or antigenome. Additional proteins may be included as desired, each encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome. Expression of the genome or antigenome and proteins from transfected plasmids can be achieved, for example, by each cDNA being under the control of a promoter

for T7 RNA polymerase, which in turn is supplied by infection, transfection or transduction with an expression system for the T7 RNA polymerase, e.g., a vaccinia virus MVA strain recombinant which expresses the T7 RNA polymerase (Wyatt et al., *Virology*, 210: 202-205 (1995), incorporated herein by reference in its entirety). The viral proteins, and/or T7 RNA polymerase, can also be provided by transformed mammalian cells or by transfection of preformed mRNA or protein.

A PIV antigenome may be constructed for use in the present invention by, e.g., assembling cloned cDNA segments, representing in aggregate the complete antigenome, by polymerase chain reaction or the like (PCR; described in, e.g., U.S. Patent Nos. 4,683,195 and 4,683,202, and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, San Diego (1990), each incorporated herein by reference in its entirety) of reverse-transcribed copies of PIV mRNA or genome RNA. For example, a first construct is generated which comprises cDNAs containing the left hand end of the antigenome, spanning from an appropriate promoter (e.g., T7 RNA polymerase promoter) and assembled in an appropriate expression vector, such as a plasmid, cosmid, phage, or DNA virus vector. The vector may be modified by mutagenesis and/or insertion of synthetic polylinker containing unique restriction sites designed to facilitate assembly. For ease of preparation the N, P, L and other desired PIV proteins can be assembled in one or more separate vectors. The right hand end of the antigenome plasmid may contain additional sequences as desired, such as a flanking ribozyme and tandem T7 transcriptional terminators. The ribozyme can be hammerhead type (e.g., Grosfeld et al., (1995), *supra*), which would yield a 3' end containing a single nonviral nucleotide, or can be any of the other suitable ribozymes such as that of hepatitis delta virus (Perrotta et al., *Nature* 350:434-436 (1991), incorporated herein by reference in its entirety) which would yield a 3' end free of non-PIV nucleotides. The left- and righthand ends are then joined via a common restriction site.

A variety of nucleotide insertions, deletions and rearrangements can be made in the PIV genome or antigenome during or after construction of the cDNA. For example, specific desired nucleotide sequences can be synthesized and inserted at appropriate regions in the cDNA using convenient restriction enzyme sites. Alternatively, such techniques as site-specific mutagenesis, alanine scanning, PCR mutagenesis, or other such techniques well known in the art can be used to introduce mutations into the cDNA.

Alternative means to construct cDNA encoding the genome or antigenome include reverse transcription-PCR using improved PCR conditions (e.g., as described in Cheng et al., Proc. Natl. Acad. Sci. USA 91:5695-5699 (1994)), incorporated herein by reference) to reduce the number of subunit cDNA components to as few as one or two pieces. In other embodiments different promoters can be used (e.g., T3, SP6) or different ribozymes (e.g., that of hepatitis delta virus). Different DNA vectors (e.g., cosmids) can be used for propagation to better accommodate the larger size genome or antigenome.

Isolated polynucleotides (e.g., cDNA) encoding the genome or antigenome may be inserted into appropriate host cells by transfection, electroporation, mechanical insertion, transduction or the like, into cells which are capable of supporting a productive PIV infection, e.g., HEp-2, FRhL-DBS2, LLC-MK2, MRC-5, and Vero cells. Transfection of isolated polynucleotide sequences may be introduced into cultured cells by, for example, calcium phosphate-mediated transfection (Wigler et al., Cell 14: 725 (1978); Corsaro and Pearson, Somatic Cell Genetics 7: 603 (1981); Graham and Van der Eb, Virology 52: 456 (1973)), electroporation (Neumann et al., EMBO J. 1: 841-845 (1982)), DEAE-dextran mediated transfection (Ausubel et al., (ed.) Current Protocols in Molecular Biology, John Wiley and Sons, Inc., NY (1987)), cationic lipid-mediated transfection (Hawley-Nelson et al., Focus 15: 73-79 (1993)) or a commercially available transfection reagent, e.g., LipofectACE® (Life Technologies, Gaithersburg, MD) or the like.

(each of the foregoing references are incorporated herein by reference in its entirety).

As noted above, in some embodiments of the invention the N, P, L and other desired PIV proteins are encoded by one or more helper viruses which is phenotypically distinguishable from that which encodes the genome or antigenome. The N, P, L and other desired PIV proteins can also be encoded by one or more expression vectors which can be the same or separate from that which encodes the genome or antigenome, and various combinations thereof. Additional proteins may be included as desired, encoded by its own vector or by a vector encoding one or more of the N, P, L and other desired PIV proteins, or the complete genome or antigenome.

The compositions and methods of the invention permit analysis and manipulation of PIV molecular biology and pathogenic mechanisms using, e.g., defined mutations to alter the function or expression of selected PIV proteins. Using these methods and compositions, one can readily distinguish mutations responsible for desired phenotypic changes from silent incidental mutations, and select phenotype-specific mutations for incorporation into a recombinant PIV genome or antigenome for vaccine production.

Modifications of PIV provided within the invention are directed toward the production of improved vaccine viruses, e.g., to enhance viral attenuation and vaccine immunogenicity, to ablate epitopes associated with undesirable immunopathology, to accommodate antigenic drift, etc. To achieve these and other objectives, the compositions and methods of the invention allow for a wide variety of modifications to be introduced into a PIV genome or antigenome for incorporation into infectious, recombinant PIV. For example, foreign genes or gene segments encoding protective antigens or epitopes may be added within a PIV clone to generate PIV virus strains capable of inducing immunity to both PIV and another virus or agent from which the protective antigen was derived. Alternatively, foreign genes may be inserted, in whole or in part, encoding modulators of the

immune system, such as cytokines, to enhance immunogenicity of a vaccine virus.

Other mutations which may be included within PIV clones of the invention include, for example, substitution of heterologous genes or gene segments (e.g., a gene segment encoding a cytoplasmic tail of a glycoprotein gene) with a counterpart gene or gene segment in a PIV clone.

Alternatively, the relative order of genes within a PIV clone can be changed, the PIV genome promoter can be replaced with its antigenome counterpart, or selected gene(s) rendered non-functional (e.g., by functional ablation involving introduction of a stop codon to prevent expression of the gene). Other modifications in a PIV clone can be made to facilitate manipulations, such as the insertion of unique restriction sites in various non-coding or coding regions or elsewhere. In addition, nontranslated gene sequences can be removed to increase capacity for inserting foreign sequences.

Thus, by providing infectious clones of PIV the invention permits a wide range of alterations to be recombinantly produced within the PIV genome (or antigenome), yielding defined mutations which specify desired phenotypic changes. By "infectious clone" is meant cDNA or its product, synthetic or otherwise, RNA capable of being directly incorporated into infectious virions which can be transcribed into genomic or antigenomic RNA capable of serving as a template to produce the genome of infectious viral or subviral particles. As noted above, defined mutations can be introduced by a variety of conventional techniques (e.g., site-directed mutagenesis) into a cDNA copy of the genome or antigenome. The use of genomic or antigenomic cDNA subfragments to assemble a complete genome or antigenome cDNA as described herein has the advantage that each region can be manipulated separately, where small cDNA subjects provide for better ease of manipulation than large cDNA subjects, and then readily assembled into a complete cDNA. Thus, the complete antigenome or genome cDNA, or a selected subfragment thereof, can be used as a template for oligonucleotide-directed mutagenesis. This can be through the intermediate of a

single-stranded phagemid form, such as using the MUTA-gen® kit of Bio-Rad Laboratories (Richmond, CA), or a method using the double-stranded plasmid directly as a template such as the Chameleon® mutagenesis kit of Strategene (La Jolla, CA), or by
5 the polymerase chain reaction employing either an oligonucleotide primer or a template which contains the mutation(s) of interest. A mutated subfragment can then be assembled into the complete antigenome or genome cDNA. A variety of other mutagenesis techniques are known and can be
10 routinely adapted for use in producing the mutations of interest in a PIV antigenome or genome cDNA of the invention.

Thus, in one illustrative embodiment mutations are introduced by using the MUTA-gene® phagemid in vitro mutagenesis kit available from Bio-Rad Laboratories. In brief, cDNA encoding an PIV genome or antigenome is cloned into the plasmid pTZ18U, and used to transform CJ236 cells (Life Technologies). Phagemid preparations are prepared as recommended by the manufacturer. Oligonucleotides are designed for mutagenesis by introduction of an altered nucleotide at the desired position of the genome or antigenome. The plasmid containing the genetically altered genome or antigenome is then amplified.
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Mutations can vary from single nucleotide changes to the introduction, deletion or replacement of large cDNA segments containing one or more genes or genome segments. Genome segments can correspond to structural and/or functional domains, e.g., cytoplasmic, transmembrane or ectodomains of proteins, active sites such as sites that mediate binding or other biochemical interactions with different proteins,
25 epitopic sites, e.g., sites that stimulate antibody binding and/or humoral or cell mediated immune responses, etc. Useful genome segments in this regard range from about 15-35 nucleotides in the case of genome segments encoding small functional domains of proteins, e.g., epitopic sites, to about
30 50, 75, 100, 200-500, and 500-1,500 or more nucleotides.
35

The ability to introduce defined mutations into infectious PIV has many applications, including the manipulation of PIV pathogenic and immunogenic mechanisms.

For example, the functions of PIV proteins, including the N, P, M, F, HN, and L proteins and C, D and V ORF products, can be manipulated by introducing mutations which ablate or reduce the level of protein expression, or which yield mutant
5 protein. In one such exemplary modification, a sequence at the cleavage site of the F protein can be modified and the effects of this modification on growth in tissue culture and infection and pathogenesis of the resultant PIV can be routinely determined in experimental animals.

10 Various genome RNA structural features, such as promoters, intergenic regions, and transcription signals, can also be routinely manipulated within the methods and compositions of the invention. The effects of trans-acting proteins and cis-acting RNA sequences can be readily
15 determined, for example, using a complete antigenome cDNA in parallel assays employing PIV minigenomes (Dimock, et al., J. Virol. 67: 2772-8 (1993), incorporated herein by reference in its entirety), whose rescue-dependent status is useful in characterizing those mutants that may be too inhibitory to be recovered in replication-independent infectious virus.
20

The present invention further provides tools and methods to readily distinguish between silent incidental mutations and mutations responsible for phenotype differences, for example by introducing suspect mutations, separately and
25 in various combinations, into the genome or antigenome of infectious wild-type (i.e., for one or more phenotypic character such as temperature sensitivity, replication in a selected host, etc.) PIV. This process permits identification of mutations responsible for desired vaccine phenotypes such
30 as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc. Mutations identified by these methods can then be introduced in various combinations to modify a vaccine virus to an appropriate level of attenuation, etc., as desired. Moreover, the present
35 invention provides the ability to combine mutations from different strains of virus into a single vaccine strain.

As noted above, mutations incorporated within recombinantly altered PIV clones may be selected based on

their ability to alter expression and/or function of a selected PIV protein, yielding a desired phenotypic change, or for a variety of other purposes. Desired phenotypic changes include, e.g., changes in viral growth in culture, temperature sensitivity, plaque size, attenuation, and immunogenicity.

5 For example, a polynucleotide sequence encoding the genome or antigenome can be modified by a nucleotide insertion, rearrangement, deletion or substitution to specify attenuation, temperature-sensitivity, cold-adaptation, small

10 plaque size, host range restriction, alteration in gene expression, or a change in an immunogenic epitope.

Sub-D

~~In one aspect of the invention, mutations occurring in biologically derived, attenuated PIV are identified and introduced individually or in combination into a full-length PIV clone, and the phenotypes of rescued recombinant viruses containing the introduced mutations are determined. In exemplary embodiments, amino acid changes displayed by biologically derived mutant viruses over a wild-type PIV, for example changes exhibited by PIV mutants having ts, ca or att phenotypes, are incorporated within recombinant PIV clones. These changes from biologically derived mutant PIV specify desired characteristics in the resultant clones, e.g., an attenuation phenotype specified by a mutation adopted from the HPIV3 mutant US cp45. These changes are preferably introduced into recombinant virus using two or three nucleotide changes compared to a corresponding wild type or biologically derived mutant sequence, which has the effect of stabilizing the mutation against genetic reversion.~~

The present invention also provides recombinant PIV having multiple, phenotype-specifying mutations introduced in selected combinations into the genome or antigenome of an infectious clone. This process, coupled with evaluation of phenotype, provides mutant recombinant PIV having such desired characteristics as attenuation, temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc. Thus, exemplary PIV clones are disclosed herein which incorporate one or more, and preferably at least two attenuating mutations, e.g., ts, ca or att mutations adopted

from a biologically derived PIV mutant, such as JS cp45. Target genes for adopting biologically derived mutations in a recombinant PIV in this context include the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F and the C, D and V ORF products. Also targeted are extragenic sequences, e.g., sequences in the 3' leader or trailer regions of a PIV genome, and in cis-acting elements such as gene start and gene end sequences, e.g., the N gene start signal. Exemplary mutations incorporated in recombinant PIV herein include one or more nucleotide substitutions specifying amino acid change(s) in the polymerase L gene, e.g., at Tyr₉₄₂, Leu₉₉₂, and/or Thr₁₅₅₈. For example, PIV recombinants are disclosed wherein Tyr₉₄₂ is replaced by His, Leu₉₉₂ is replaced by Phe, and/or Thr₁₅₅₈ is replaced by Ile. These mutations have been successfully incorporated in various exemplary PIV recombinants herein, including r942, r992, r1558, r942/992, r992/1558, r942/1558, or r942/992/1558 recombinants described in the Examples below.

Other exemplary mutations adopted from a biologically derived PIV mutant include one or more mutations in the N protein, including specific mutations at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS cp45. In more detailed aspects, these mutations are represented as Val₉₆ to Ala or Ser₃₈₉ to Ala. Also disclosed in the Examples below are recombinant PIV which encode an amino acid substitution in the C protein, e.g., a mutation at a position corresponding to Ile₉₆ of JS cp45, preferably represented by a substitution of Ile₉₆ to Thr. Further exemplary mutations adopted from biologically derived PIV mutants include one or more mutations in the F protein, including mutations adopted from JS cp45 at a position corresponding to residues Ile₄₂₀ or Ala₄₅₀ of JS cp45, preferably represented by acid substitutions Ile₄₂₀ to Val or Ala₄₅₀ to Thr. Other PIV recombinants within the invention adopt one or more amino acid substitutions in the HN protein, as exemplified hereinbelow by a recombinant PIV adopting a mutation at a position corresponding to residue Val₃₈₄ of JS cp45, preferably represented by the substitution Val₃₈₄ to Ala.

Yet additional examples within this aspect of the invention include recombinant PIV which incorporate one or more mutations in an extragenic sequence, e.g., a 3' leader sequence of recombinant PIV genome or antigenome. Exemplary mutations in this context include mutations in the 3' leader occurs at one or more positions corresponding to nucleotide 23, 24, and/or 28 of JS cp45, for example a T to C change at nucleotide 23, a C to T change at nucleotide 24, or a G to T change at nucleotide 28. Yet additional extragenic mutations include one or more mutations in a N gene start sequence, as exemplified hereinbelow by a mutation in the N gene start sequence at a position corresponding to nucleotide 62 of JS cp45, preferably represented by a A to T change. The above exemplary mutations adopted from biologically derived mutant PIV are evaluated and combined into recombinant PIV in the Examples below to result, individually and/or in combination, in novel, attenuated candidate vaccine strains, as exemplified by the recombinants designated herein as rcp45, rcp45 3'NCMFHN, rcp45 3'NL, rcp45 3'N, and rcp45 F. Other PIV recombinants within the invention will incorporate a plurality and up to a full complement of the mutations present in one or more of these exemplary recombinants, as well as mutations identified in other biologically derived mutant PIV strains identified and adopted in a recombinant PIV according to the teachings herein.

Mutations identified according to the methods disclosed herein are compiled into a "menu" and introduced in various combinations to calibrate a vaccine virus to a selected level of attenuation, immunogenicity and stability.

In preferred embodiments, the invention provides for supplementation of one or more mutations adopted from biologically derived PIV, e.g., ts, ca or att mutations, with additional types of mutations involving the same or different genes. Target genes for mutation in this context also include the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F and the C, D and V ORF products. In one aspect, recombinant PIVs are provided wherein at least

one attenuating mutation occurs in the PIV polymerase gene L and involves a nucleotide substitution specifying a *ts* or *att* phenotype adopted from a biologically derived mutant PIV strain, for example JS cp45. Exemplary HPIV3 recombinants disclosed herein include the r942, r992, r1558, r942/992, r992/1558, r942/1558, or r942/992/1558 recombinants described in the Examples below. These exemplary PIV clones incorporate one or more nucleotide substitutions resulting in an amino acid change in the polymerase gene, e.g., at Tyr₉₄₂, Leu₉₉₂, and/or Thr₁₅₅₈. For example, PIV recombinants are disclosed wherein Tyr₉₄₂ is replaced by His, Leu₉₉₂ is replaced by Phe, and/or Thr₁₅₅₈ is replaced by Ile. Preferably, two or three mutations are incorporated in a codon specifying an attenuating mutation adding increased stability against phenotypic reversion.

In additional aspects, a variety of other genetic alterations can be produced in a recombinant PIV genome or antigenome for incorporation into infectious recombinant PIV, alone or together with one or more attenuating mutations adopted from a biologically derived mutant PIV. Heterologous genes (e.g. from different PIV strains or non-PIV sources such as another virus, e.g., RSV or measles virus) may be inserted or substituted, in whole or in part, the order of genes changed, gene overlap removed, the PIV genome promoter replaced with its antigenome counterpart, and even entire, non-essential genes deleted. In one aspect, a selected PIV gene, for example the C, D, or V ORF, is functionally deleted to yield a recombinant PIV having novel phenotypic characteristics, for example enhanced growth *in vitro* and/or attenuation *in vivo*. An infectious PIV clone of the invention can also be engineered to enhance its immunogenicity and induce a level of protection greater than that provided by natural infection, or to ablate epitopes associated with undesirable immunopathologic reactions. Enhanced immunogenicity of the vaccines produced by the present invention addresses one of the greatest obstacles to controlling PIV, namely the incomplete nature of immunity induced by natural infection. In this context, additional

gene(s) or gene segment(s) may be inserted into or proximate to the PIV genome or antigenome which may be placed under the control of a common or independent set of transcription signals. Genes of interest include those encoding cytokines (e.g., IL-2 through IL-15, especially IL-2, IL-6 and IL-12, etc.) and proteins rich in T helper cell epitopes. The additional protein can be expressed either as a separate protein or as a chimera engineered from a second copy of one of the PIV proteins, such as HN. This provides the ability to modify and improve the immune response against PIV both quantitatively and qualitatively.

Other mutations useful within the invention involve replacement of the 3' end of genome with its counterpart from antigenome, which is associated with changes in RNA replication and transcription. In addition, intergenic regions can be shortened or lengthened or changed in sequence content. In yet additional aspects, PIV useful in a vaccine formulation can be conveniently modified to accommodate antigenic drift in circulating virus. Typically the modification will be in the HN and/or F proteins. For example, a selected antigenic form of an entire HN or F gene, or the segment(s) encoding particular immunogenic regions thereof, is incorporated into a PIV genome or antigenome cDNA by replacement of a counterpart region in the infectious clone, or by adding one or more copies of the gene such that several antigenic forms are represented in the resultant clone. Progeny virus produced from the modified PIV cDNA are then used in vaccination protocols against emerging strains.

Other mutations for use in infectious PIV of the invention include mutations in cis-acting signals identified during mutational analysis of PIV minigenomes. For example, insertional and deletional analysis of leader and trailer and flanking sequences identify viral promoters and transcription signals and provide a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide alternatives) of these cis-acting signals also identifies mutations which

reduce or increase RNA replication or transcription. Any of these mutations can be inserted into the complete antigenome or genome as described herein.

Additional modifications in PIV clones can be made to facilitate manipulations, such as the insertion of unique restriction sites in various intergenic regions or elsewhere. Nontranslated gene sequences can also be removed to increase capacity for inserting foreign sequences.

Certain substitutions, insertions, deletions or rearrangements of genes or gene segments within recombinant PIV of the invention (e.g., substitutions of a gene segment encoding a selected protein or protein region, for instance a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc.) are made in structural or functional relation to an existing, "counterpart" gene or gene segment from the same or different PIV or other source. Such modifications yield novel recombinants having desired phenotypic changes compared to wild-type or parental PIV or other viral strains. For example, recombinants of this type may express a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of another PIV. Other exemplary recombinants of this type express duplicate protein regions, such as duplicate immunogenic regions.

As used herein, "counterpart" genes, gene segments, proteins or protein regions, are typically from heterologous sources (e.g., from different PIV genes, or representing the same (i.e., homologous or allelic) gene or gene segment in different PIV types or strains). Typical counterparts selected in this context share gross structural features, e.g., each counterpart may encode a comparable protein or protein structural domain, such as a cytoplasmic domain, transmembrane domain, ectodomain, binding site or region, epitopic site or region, etc. Counterpart domains and their encoding gene segments embrace an assemblage of species having a range of size and sequence variations defined by a common biological activity among the domain or gene segment variants.

For example, two selected protein domains encoded by counterpart gene segments within the invention share substantially the same qualitative activity, such as providing a membrane spanning function, a specific binding activity, an 5 immunological recognition site, etc. More typically, a specific biological activity shared between counterparts, e.g., between selected protein segments or proteins, will be substantially similar quantitatively, i.e., they will not vary in respective quantitative activity levels by more than 30%, 10 preferably by no more than 20%, more preferably by no more than 5-10%.

Counterpart genes and gene segments, as well as other polynucleotides disclosed herein for producing recombinant PIV within the invention, preferably share substantial sequence identity with a selected polynucleotide "reference sequence," e.g., with another selected counterpart sequence. As used herein, a "reference sequence" is a defined sequence used as a basis for sequence comparison, for example, a segment of a full-length cDNA or gene, or a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since 15 two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences 20 of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A 25 "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison 30 window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal 35

alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, Adv. Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. USA 85:2444 (1988) (each of which is incorporated by reference), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI, incorporated herein by reference), or by inspection, and the best alignment (i.e., resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window

of comparison. The reference sequence may be a subset of a larger sequence.

In addition to these polynucleotide sequence relationships, proteins and protein regions encoded by recombinant PIV of the invention are also typically selected to have conservative relationships, i.e. to have substantial sequence identity or sequence similarity, with selected reference polypeptides. As applied to polypeptides, the term "sequence identity" means peptides share identical amino acids at corresponding positions. The term "sequence similarity" means peptides have identical or similar amino acids (i.e., conservative substitutions) at corresponding positions. The term "substantial sequence identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). The term "substantial similarity" means that two peptide sequences share corresponding percentages of sequence similarity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. Abbreviations for the twenty naturally occurring amino acids used herein follow conventional usage

(Immunology - A Synthesis (2nd ed., E.S. Golub & D.R. Gren, eds., Sinauer Associates, Sunderland, MA, 1991), incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). Moreover, amino acids may be modified by glycosylation, phosphorylation and the like.

The infectious PIV produced from cDNA-expressed genome or antigenome can be any of the PIV or PIV-like strains, e.g., human, bovine, murine, etc. To engender a protective immune response, the PIV strain may be one which is endogenous to the subject being immunized, such as human PIV being used to immunize humans. The genome or antigenome can be modified, however, to express heterologous PIV genes or gene segments, or genes or gene segments from other heterologous sources, e.g., RSV or measles virus. Thus, infectious PIV intended for administration to humans may be human PIV that has been modified to contain genes or gene segments from a bovine or murine PIV type such as for the purpose of attenuation. BPIV3 possesses host range mutations that restrict its replication in rhesus monkeys and humans (Karron et al., supra, 1995a; van Wyke Coelingh et al., 1988), each incorporated herein by reference in its entirety). Gene(s), mutations and cis-acting regulatory sequences of BPIV3 that specify desired phenotypes, e.g., host range restriction, will be identified by their substitution for corresponding sequences in rPIV of the invention, and incorporated within further modified rPIV to develop yet additional useful vaccine agents. Similarly, mutations of JS cp45 which are known to impart non-ts host-range attenuating mutations for the rhesus monkey (Hall et al., supra, (1992))

will likewise be identified and incorporated into modified rPIV vaccine agents of the invention. Alternatively, a bovine PIV may be modified to contain genes that encode, e.g., proteins or immunogenic epitopes that elicit protection
5 against human PIV infection. For example, human PIV glycoprotein genes can be substituted for counterpart bovine glycoprotein genes, such that the bovine PIV elicits a protective immune response in humans against human PIV strains.

In exemplary embodiments, individual genes, gene segments, or single or multiple nucleotides of one PIV are substituted by counterpart sequence(s) from a heterologous PIV or other source. For example, heterologous gene segments, such as one encoding a cytoplasmic tail, transmembrane domain or ectodomain, an epitopic site or region, a binding site or region, an active site or region containing an active site, etc., of a selected protein from one PIV is substituted for a counterpart gene segment in another PIV to yield novel recombinants, for example recombinants expressing a chimeric protein having a cytoplasmic tail and/or transmembrane domain of one PIV fused to an ectodomain of another PIV. Preferred genome segments in this regard range from about 15-35 nucleotides in the case of gene segments encoding small functional domains of proteins, e.g., epitopic sites, to about 20 50, 75, 100, 200-500, or 500-1,500 or more nucleotides for 25 gene segments encoding larger domains or protein regions.

In one aspect of the invention, selected domains of HN and/or F proteins of one PIV strain are substituted into a heterologous PIV clone to produce a recombinant virus capable 30 of stimulating a cross-protective immune response against both PIV strains in an immunized host. In other aspects, modified PIV clones are provided which comprise a chimera of a human PIV genomic or antigenomic sequence and at least one non-human PIV sequence, for example a polynucleotide containing 35 sequences from both human and bovine PIV. The replacement of a human PIV coding sequence or non-coding sequence (e.g., a promoter, gene-end, gene-start, intergenic or other cis-acting element) with a counterpart bovine or murine PIV sequence

yields recombinants having a variety of possible attenuating effects. For example, a host range effect will often arise from a heterologous PIV gene not functioning efficiently in a human cell, from incompatibility of the heterologous sequence or protein with a biologically interactive human PIV sequence or protein (e.g., a sequence or protein that ordinarily cooperates with the substituted sequence or protein for viral transcription, translation, assembly, etc.), among other useful attenuating effects. In yet another aspect of the invention, insertion of foreign genes or gene segments, and in some cases of noncoding nucleotide sequences, into the PIV genome results in a desired increase in genome length causing yet additional, desired phenotypic effects. Increased genome length is expected to result in attenuation of the resultant PIV clone, dependent in part upon the length of the insert. In addition, the expression of certain proteins from a gene inserted into recombinant PIV will result in attenuation of the virus due to the action of the protein. This has been described for IL-2 expressed in vaccinia virus (see, e.g., Flexner et al., Nature 33:259-62 (1987)) and also would be expected for gamma interferon.

Deletions, insertions, substitutions and other mutations involving changes of whole viral genes or gene segments in recombinant PIV of the invention yield highly stable vaccine candidates, which are particularly important in the case of immunosuppressed individuals. Many of these mutations will result in attenuation of resultant vaccine strains, whereas others will specify different types of desired phenotypic changes. For example, certain viral genes are known which encode proteins that specifically interfere with host immunity (see, e.g., Kato et al., EMBO J. 16:578-87 (1997), incorporated herein by reference). Ablation of such genes in vaccine viruses is expected to reduce virulence and pathogenesis and/or improve immunogenicity.

In preferred aspects of the invention, the modified PIV clones represent a chimera of two or more human PIV genomes, for example a clone containing polynucleotide sequences from HPIV3 joined to sequences from one or more

heterologous human PIV, such as HPIV1 and HPIV2. Thus, individual genes or gene segments of human PIV3 may be replaced or supplemented with counterpart genes or gene segments from HPIV 1 or HPIV2, or visa versa. In one example described hereinbelow, the invention provides a PIV clone, rPIV3-1, into which both the HN and F glycoprotein genes of HPIV1 are substituted for their counterpart genes in an HPIV3 background, yielding a chimeric virus having immunological characteristics representative of both parental strains.

In additional aspects of the invention, chimeric PIV or PIV clones having other alterations of genes or gene segments, as described above, are further modified by introducing one or more attenuating mutations adopted from a biologically derived mutant PIV, e.g., HPIV3 JS cp45 to achieve an attenuated, or further attenuated, chimeric mutant derivative. For example, one or more human PIV coding or non-coding polynucleotides may be substituted with a counterpart sequence from a heterologous human PIV, bovine PIV or murine PIV as described above, and this alteration may be combined with one or more mutations specifying, e.g., a ts, ca or att phenotype adopted from a biologically derived attenuated PIV mutant, to yield an attenuated or further attenuated (i.e., compared to either the chimeric clone or biologically derived parent virus) vaccine virus. Alternatively, functional deletion of a non-essential gene or gene segment, such as the C, D or V ORF, may be combined in a recombinant PIV with one or more mutations specifying a ts, ca or att phenotype from biologically derived PIV mutants to yield an attenuated vaccine strain. These combinatorial modifications yield recombinant PIV having desired phenotypic characteristics, e.g., increased yield of virus, enhanced attenuation, and/or genetic resistance to reversion from an attenuated phenotype, due to the combined effects of the different selected mutations.

In one combinatorial mutation design, a modified PIV is provided which comprises a chimera of a human PIV genomic or antigenomic sequence and at least one non-human PIV sequence, for example a polynucleotide containing sequences

from both human and bovine PIV, and which also incorporates one or more mutations adopted from biologically derived PIV, e.g., one or more naturally occurring *ts*, *ca* or *att* mutations. Alternatively, the modified PIV can be a chimera of two or

5 more human PIV genomes, for example a polynucleotide containing sequences from HPIV3 joined to sequences from one or more heterologous human PIVs, such as HPIV1 and HPIV2, which further incorporates one or more *ts*, *ca* *att* or other selected mutations from biologically derived PIV (e.g., a

10 nucleotide substitution specifying a *ts*, *ca* or *att* phenotype adopted from a biologically derived mutant PIV strain such as JS cp45). In more detailed aspects, individual genes or gene segments of human PIV3 are replaced or supplemented with counterpart genes or gene segments from HPIV1 or HPIV2, or visa versa, in a clone that is attenuated or further attenuated by, e.g., a nucleotide change encoding an amino acid substitution conferring a *ts* mutation in the large polymerase L gene. For example, the invention provides PIV clones having the HN and/or F glycoprotein genes of HPIV1 substituted for their counterpart genes in an HPIV3 background, wherein the phenotype of the resultant chimeric clone is further modified by *ts*, *ca* or *att* mutation(s) encoded within one or more of the N, P, L, M, HN, F, C, D and V genes. Various combinations from a menu of possible mutations

15 disclosed herein can be made to calibrate a vaccine virus to a selected level of attenuation, immunogenicity and stability, e.g., to achieve a satisfactorily attenuated and immunogenic, chimeric virus having immunological characteristics representative of multiple PIV strains. In one aspect,

20 recombinant PIVs are provided wherein at least one attenuating mutation occurs in the PIV polymerase gene L (as exemplified by the recombinants r942, r992, r1558, r942/992, r942/1558, r992/1558, or r942/992/1558 described in the Examples below) incorporated in a chimeric PIV background. For example,

25 useful chimeric PIV recombinants within this aspect of the invention will have one or more genes or gene segments of the HN and/or F glycoprotein genes from, e.g., HPIV1 substituted for their counterpart gene(s) in a heterologous background,

e.g., in an HPIV3 clone, and will further incorporate one or more attenuating mutations, eg., nucleotide substitutions resulting in an amino acid change in the polymerase gene (such as change from Tyr to His at position 942, a change from Leu to Phe at position 992, and/or a change from Thr to Ile at position 1558) from a biologically derived PIV mutant. One such chimeric, attenuated recombinant exemplified hereinbelow is rPIV3-1.cp45L, a derivative of rPIV3-1 which incorporates all three L gene mutations specified above from JS cp45.

Yet additional mutations which can be incorporated in a chimeric PIV background for developing vaccine strains will be selected from biologically derived mutations in other genes, or will be created *de novo* in a recombinant genome by standard site directed mutagenesis or other purely recombinant mutagenic methods. Target genes for adopting biologically derived mutations or creating novel mutations in a recombinant PIV in this context include the nucleocapsid protein N, phosphoprotein P, large polymerase subunit L, matrix protein M, hemagglutinin-neuraminidase protein HN, fusion protein F and the C, D and V ORF products. Also targeted are extragenic sequences, eg., sequences in the 3' leader or trailer regions of a PIV genome. Exemplary mutations identified and incorporated in non-chimeric, recombinant PIV, described above will thus be readily incorporated within a chimeric PIV background, eg., as exemplified by rPIV3-1. These exemplary mutations include one or more mutations in the N protein, including specific mutations at a position corresponding to residues Val₉₆ or Ser₃₈₉ of JS cp45. In more detailed aspects, these mutations are represented as Val₉₆ to Ala or Ser₃₈₉ to Ala. Also desired for incorporation in chimeric PIV recombinants are mutations in the C protein, eg., a mutation at a position corresponding to Ile₉₆ of JS cp45, preferably represented by a substitution of Ile₉₆ to Thr, as described above. Further exemplary mutations for incorporation in a chimeric PIV background include one or more mutations in the F protein, for example adopted from JS cp45 at a position corresponding to residues Ile₄₂₀ or Ala₄₅₀, eg., substitutions Ile₄₂₀ to Val or Ala₄₅₀ to Thr. Yet additional chimeric PIV

recombinants within the invention will adopt one or more amino acid substitutions in the HN protein, for example a mutation at a position corresponding to residue Val₃₈₄ of JS cp45, such as Val₃₈₄ to Ala. Yet additional chimeric recombinants will incorporate one or more mutations in an extragenic sequence, eg., a 3' leader sequence of the recombinant genome or antigenome. Exemplary mutations in this context include mutations in the 3' leader occurs at one or more positions corresponding to nucleotide 23, 24, 28, and/or 45 of JS cp45, for example a T to C change at nucleotide 23, a C to T change at nucleotide 24, a G to T change at nucleotide 28, or a T to A change at nucleotide 45. Yet additional extragenic mutations for incorporation within a chimeric PIV background include one or more mutations in a N gene start sequence, as exemplified herein by a mutation in the N gene start sequence at a position corresponding to nucleotide 62 of JS cp45, such as a A to T change. These exemplary mutations evaluated and combined into recombinant PIV in the Examples below will be readily incorporated within a chimeric PIV recombinant using the methods and tools provided herein, and will specify, individually and/or in combination, desired phenotypic changes to yield yet additional attenuated chimeric vaccine strains within the invention.

In additional combinatorial mutation designs, modified PIVs are provided which incorporate one or more of the foregoing ts, ca or att mutations adopted from biologically derived PIV or generated recombinantly in a PIV clone of the invention, in combination with another, distinct mutation disclosed herein (e.g., a deletion, addition, or rearrangement of a PIV N, P, L, M, HN, F, C, D or V gene or gene segment, or a gene or gene segment from another source such as RSV or measles virus). Also in this case, various combinations from a menu of mutations disclosed herein can be made to calibrate the vaccine virus to a selected level of attenuation, immunogenicity and stability. Thus, recombinant PIVs are provided which exhibit at least one attenuating mutation from a biologically derived PIV mutant, e.g., a mutation in the PIV polymerase gene L as found in JS cp45, or a recombinantly

generated mutation, and which further incorporates one or more additional changes selected from, e.g., substitution or introduction of a heterologous gene or gene segment from a non-PIV source (e.g., an immunogenic RSV or measles gene or epitope, or a gene encoding a cytokine), a change in the order of viral genes to alter expression levels, removal of gene overlap, substitution of a PIV genome promoter with its antigenome counterpart, shortening, lengthening or removal of intergenic regions, e.g., to increase capacity for inserting foreign sequences, mutations in cis-acting signals to reduce or increase RNA replication or transcription, insertion of unique restriction sites, or deletion of even entire, non-essential genes, among other changes.

For vaccine use, virus produced according to the present invention can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4°C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., saline or comprising SPG, Mg⁺⁺ and HEPES, with or without adjuvant, as further described below.

PIV vaccines of the invention contain as an active ingredient an immunogenically effective amount of PIV produced as described herein. The modified virus may be introduced into a host with a physiologically acceptable carrier and/or adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's

adjuvant, MPL™ (3-o-deacylated monophosphoryl lipid A; RIBI ImmunoChem Research, Inc., Hamilton, MT) and IL-12 (Genetics Institute, Cambridge MA), among many other suitable adjuvants well known in the art.

Upon immunization with a PIV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for PIV virus proteins, e.g., F and HN glycoproteins. As a result of the vaccination with an immunogenically effective amount of PIV produced as described herein, the host becomes at least partially or completely immune to PIV infection, or resistant to developing moderate or severe PIV infection, particularly of the lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by PIV or a closely related virus and which host is capable of generating a protective immune response to the antigens of the vaccinating strain. Accordingly, the invention provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the PIV of the invention are administered to a host susceptible to or otherwise at risk for PIV infection to enhance the host's own immune response capabilities. Such an amount is defined to be a "immunogenically effective dose." In this use, the precise amount of PIV to be administered within an effective dose will depend on the host's state of health and weight, the mode of administration, the nature of the formulation, etc., but will generally range from about 10^3 to about 10^6 plaque forming units (PFU) or more of virus per host, more commonly from about 10^4 to 10^5 PFU virus per host. In any event, the vaccine formulations should provide a quantity of modified PIV of the invention sufficient to effectively protect the host patient against serious or life-threatening PIV infection.

The PIV produced in accordance with the present invention can be combined with viruses of other PIV serotypes or strains to achieve protection against multiple PIV

serotypes or strains. Alternatively, protection against multiple PIV serotypes or strains can be achieved by combining protective epitopes of multiple serotypes or strains engineered into one virus, as described herein. Typically when different viruses are administered they will be in admixture and administered simultaneously, but they may also be administered separately. Immunization with one strain may protect against different strains of the same or different serotype.

In some instances it may be desirable to combine the PIV vaccines of the invention with vaccines which induce protective responses to other agents, particularly other childhood viruses. In another aspect of the invention the PIV can be employed as a vector for protective antigens of other pathogens, such as respiratory syncytial virus (RSV) or measles virus, by incorporating the sequences encoding those protective antigens into the PIV genome or antigenome which is used to produce infectious PIV, as described herein. The cloning of RSV cDNA and other disclosure relevant to the invention is described in copending United States patent applications Serial Nos. 08/534,768, 60/021,773, 08/720,132, 60/046,141, 60/047,634, and 08/892,403, and PCT patent application PCT/US97/12269, each incorporated herein by reference.

Single or multiple administrations of the vaccine compositions of the invention can be carried out. In neonates and infants, multiple administration may be required to elicit sufficient levels of immunity. Administration should begin within the first month of life, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) PIV and/or other subject viral infections. Similarly, adults who are particularly susceptible to repeated or serious PIV infection, for example health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses.

Levels of induced immunity provided by the vaccines of the invention can be monitored by measuring amounts of neutralizing secretory and serum antibodies. Based on these measurements, vaccine dosages can be adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be advantageous for different recipient groups. For example, an engineered PIV strain expressing an additional protein rich in T cell epitopes may be particularly advantageous for adults rather than for infants.

In yet another aspect of the invention the PIV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment the recombinant PIV genome or antigenome incorporates a sequence which is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different promoter from that which controls PIV expression. The infectious PIV produced by coexpressing the recombinant PIV genome or antigenome with the N, P, L and other desired PIV proteins, and containing a sequence encoding the gene product of interest, is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant PIV is administered in an amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Representative gene products which may be administered within this method are preferably suitable for transient expression, including, for example, interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and vaccine antigens.

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The following examples are provided by way of illustration, not limitation.

EXAMPLE IConstruction of Plasmid p218(131)
Encoding Negative sense PIV Genomic RNA

5 A full cDNA clone designated p218(131) (Fig. 1; SEQ
ID NO: 1) (deposited under the terms of the Budapest Treaty
with the American Type Culture Collection (ATCC) of 12301
Parklawn Drive, Rockville, Maryland 20852, U.S.A., and granted
the designation 97991) was constructed to encode the complete
10 15462 nt genomic sequence of HPIV3 JS strain. A hepatitis
delta ribozyme was placed abutting the 3' end of the genomic
sequence such that self-cleavage would yield the 3' end of
HPIV3 (Perrotta and Been, Nature 350: 434-436, (1991),
incorporated herein by reference in its entirety). A T7
15 transcription terminator was placed following the delta
ribozyme. The T7 promoter was placed adjacent to the 5' end
of the genomic sequence such that the 5' terminal nucleotide
of the HPIV3 genome was the first nucleotide synthesized. In
this configuration, the cDNA encodes a complete negative-sense
20 copy of PIV3 genomic RNA containing the correct genomic
termini without any additional heterologous nucleotides.

The HPIV3 cDNA was assembled from 14 overlapping
subclones (termed A*-L, which letters in parentheses designate
individual plasmids and do not refer to specific viral genes)
25 constructed by reverse transcription (RT) and polymerase chain
reaction (PCR) of RNA isolated from virions purified by
sucrose gradient centrifugation (Stokes et al., supra, 1992;
Stokes et al., supra, 1993, each incorporated herein by
reference in its entirety). The subclones spanned the
30 following nucleotides of genomic RNA (numbered with the 3' end
designated as position 1): 1-2058 (A*), 1874-3111 (A'),
3086-5140 (C), 4348-5276 (C'), 5072-6695 (D*), 5904-8532 (E),
7806-9898 (F), 9632-10740 (F'), 9760-10955 (G), 10862-11925
(H), 11835-12868 (I), 12426-13677 (J), 13630-14496 (K), and
35 14467-15462 (L). Each fragment was cloned into pBluescript
KSII (Stratagene, La Jolla, CA) using conventional cloning
techniques and was sequenced completely.

Plasmid p(L) was then subjected to site-directed mutagenesis to introduce the T7 promoter via a single-stranded DNA intermediate according to the MUTA-GENE procedure (BioRad, Hercules, CA). The T7 promoter was positioned so that transcription initiates at the precise 5' end of the HPIV3 genome using the negative-sense mutagenic primer:

5'-AATACGACTCACTATA*ACCAAACAAGAGAAAG-3' (SEQ ID NO: 2; T7 sequences are italicized, HPIV3-specific sequences are underlined, and the 5'-end HPIV3 nucleotide, genome position 15462, is indicated by an asterisk). This modified p(L) was designated p(L*). Plasmid p(E) was modified to yield p(E*) by the same method using the negative-sense mutagenic oligonucleotide 5'-CCAAGTACTATGAGATGCTTGATT-3' (SEQ ID NO: 3) to insert three nucleotide substitutions (underlined) into the HN gene at HPIV3 position 7903, 7913, 7915 (Fig. 1). These substitutions removed an *Hga* I site, inserted a *Sca* I site, and modified amino acid 370 of the encoded HN protein such that the epitope recognized by monoclonal antibodies (mAb) 423/6 and 170/7 was ablated (van Wyke Coelingh et al., J. Virol. 61:1473-1477, (1987), incorporated herein by reference). The p(E*) to p(K) subclones were assembled in a step-wise fashion into the p(L*) plasmid to give p(E*FF'GHIJKL*) (Fig. 1A). This plasmid includes HPIV3 nucleotides 5904-15462 with the T7 promoter adjacent to nucleotide 15462 at the 5' end of the genome. Subclones p(A*) to p(E) were assembled into a second, overlapping subclone, p(A*A'CC'D*E) which contained HPIV3 nucleotides 1-8532.

Both subclones p(E*FF'GHIJKL*) and p(A*A'CC'D*E) were sequenced completely. In addition to the introduced point mutations described above, the cDNA differed from the authentic JS HPIV3 sequence (Stokes et al., supra, 1992) by a single nucleotide substitution at position 1615 which was within the N gene and caused a substitution at amino acid 506 in the encoded protein. Three other nucleotide substitutions were found at positions 10355, 11333, and 15248 in the L gene which did not change the encoded protein (Fig. 2). These three noncoding changes were retained as additional sequence markers to identify recombinant virus (designated rPIV)

derived from cDNA, and the mutation in the N gene was corrected as described later.

Subclone p(A*A'CC'D*E) was then modified to insert the hepatitis delta virus ribozyme and T7 terminator adjacent to HPIV3 position 1. An HPIV3 minigenome in which the 3' end of the HPIV3 genome (GGT↓**GGG**) (underlined) was generated through self-cleavage of a flanking hepatitis delta virus antigenomic ribozyme (shown in part in bold-type) was previously constructed (Dimock and Collins, J. Virol. **67**: 2772-2778, (1993); Perrotta and Been, supra, (1991), each incorporated herein by reference in its entirety). The ribozyme in turn was followed by a T7 transcription terminator. This minigenome cDNA was used as a template in a PCR reaction which modified the sequence adjacent to the ribozyme cleavage site to be a *Sma* I site (CCC↓GGG) and placed an *Apa*I site (GGGCC↓C) on the downstream side of the T7 terminator. The PCR product was cloned into pKSII which had been digested with *Bss*HII and made blunt-ended by filling in, yielding p218.

p218 was designed such that any sequence could be introduced into the opened *Sma* I site by blunt-end ligation and its RNA transcript would be cleaved at the delta ribozyme cut site (NNN↓GGG). The p(A*A'CC'D*E) subclone was digested with *Hga* I and *Sal* I (8533), which released the HPIV3 cDNA, and was filled in with dNTPs and T4 DNA polymerase to give blunt termini. The *Hga* I site is 10 nucleotides upstream of HPIV3 position 1 and, when digested and filled in, leaves a blunt terminus beginning with HPIV3 position 1. The modified *Hga* I-*Sal* I fragment was gel purified and cloned into the *Sma* I site of p218. The mutation in the N gene (T at nt 1615) was corrected to the JS wt sequence (A at nt 1615) (see GenBank accession #Z11575, incorporated herein by reference) using Kunkel mutagenesis (Kunkel et al., Methods Enzymol. **154**: 367-382, (1987), incorporated herein by reference in its entirety). This plasmid was designated p218(A*A'CC'D*E) (Fig. 1).

The *Xho* I-*Ngo* MI fragment of p(E*FF'GHIJKL*), which contained the T7 promoter and the HPIV3 cDNA from nucleotides 7438-15462, was cloned into the *Xho* I-*Ngo* MI window of p218(A*A'CC'D*E) (Fig. 1). This joined the two fragments of HPIV3 cDNA at HPIV3 nucleotide 7438, yielding a plasmid containing a full-length HPIV3 cDNA encoding a negative-sense genomic RNA with the above-mentioned three desired mutations in the HN gene and three incidental mutations in the L gene. The final construct, designated p218(131) (Fig. 1; SEQ ID NO: 1), was then sequenced in its entirety by automated sequencing at the NCI Frederick Cancer Research and Development Center (Frederick, MD) using the Taq DYE Deoxy Terminator cycle sequencing kit (ABI, Foster City, CA). This identified an additional change in the HN gene, namely a change of C to T in the HN gene at position 7593 which changed HN amino acid 263 from threonine to isoleucine that is also indicated in Fig. 2.

EXAMPLE II

Transcription and RNA Replication System for HPIV3

The present example describes compositions and methods for producing a reconstituted transcription and RNA replication system for human parainfluenza virus type 3 (HPIV3). This exemplary system was developed using components expressed intracellularly from transfected plasmids driven by a T7 RNA polymerase supplied by a vaccinia virus recombinant. The system is based on a negative-sense analog of HPIV3 genomic RNA in which the viral genes were deleted and replaced with a polynucleotide encoding bacterial chloramphenicol acetyl transferase (CAT). The N, P and L proteins are expressed from cotransfected plasmids so as to direct efficient transcription and RNA replication. Transcription according to this example yields subgenomic polyadenylated mRNA, which can be readily isolated, e.g., by oligo(dT) chromatography. RNA replication according to this example yields mini-antigenome and progeny minigenome, which are shown

to be encapsidated based on resistance to digestion with micrococcal nuclease.

A) Viruses and cells

5 A vaccinia virus recombinant, vTF7-3, that expresses bacteriophage T7 RNA polymerase, was provided as described by Fuerst et al. (Proc. Natl. Acad. Sci. U.S.A. 83: 8122-8126, 1986, incorporated herein by reference in its entirety). HEp-2 monolayer cultures were maintained at 37°C in 5% CO₂ with OptiMEM 1 (Life Technologies, Gaithersburg, MD) 10 supplemented with 2% fetal bovine serum (FBS), 50 µg/ml gentamicin sulfate and 2mM glutamine.

B) cDNAs

15 cDNAs corresponding to ORFs of the N, P, and L genes of the JS strain of HPIV3 (GenBank #Z11575; Stokes et al., 1992) were individually cloned into the *Nco* I-*Sal* I window of plasmid pTM-1, in which transcription is mediated by T7 RNA polymerase and translation by an internal ribosome entry site preceding the foreign ORF (Elroy-Stein et al., Proc. Natl. Acad. Sci. U.S.A. 86: 6126-6130 (1989), incorporated herein by reference in its entirety). Each gene was first modified by 20 polymerase chain reaction (PCR) to place an *Nco* I or *Nco* I-compatible site at the translational start site and a *Sal* I site on the downstream end.

25 The plasmid p(131), which is similar to p218(131) except that it lacks the hepatitis delta virus ribozyme, was used as a template for each PCR. The primers used to amplify the N ORF were CCCTATAATTCAAC**ATGTTGAGCCTATTG** (SEQ ID NO: 4; forward primer relative to positive-sense) and GATTAAA**ATGTTGGTCGACTTAGTTGCTTCC** (SEQ ID NO: 5; italics represent restriction enzyme sites, and the translational start site is in bold). The PCR product, a 1578 bp fragment flanked by an *Afl* III and *Sal* I site, was cloned into the *Nco* I-*Sal* I window of pTM-1 to yield pTM(N).

30 The primers used to amplify the PIV3 phosphoprotein (P) ORF were 5'-CCATAGAGAGT**CCATGGAAAGCGATGCTAAAACTATC-3'** (SEQ ID NO:6; forward primer) and 5'-CGGTGTCGTTCTTG**TGACTCATTGGCAATTGTTG-3'** (SEQ ID NO:7; reverse primer). A full-length cDNA of JS strain of genomic RNA

(p131) was used as template for the PCR. The resultant PCR product was an 1851 bp fragment flanked by an *Nco* I and *Sal* I restriction site (in italics). The PCR product was then cloned into the *Nco* I-*Sal* I window of pTM-1 to yield pTM(P).

5 A second PCR was performed to amplify the PIV3 phosphoprotein P ORF without the C ORF. p131 was again used as template cDNA. A different forward primer and the same reverse primer were used to amplify the PIV3 P ORF without C; 10 5'-CCATAGAGAGTCCATGGAAAGCGACGCTAAAAACTATC-3' (SEQ ID NO: 74; forward primer) and 5'-CGGTGTCGTTCTTGTCGACTCATTGGCAATTGTTG-3' (SEQ ID NO:7; reverse primer). The resultant PCR product was an 1851 bp fragment flanked by an *Nco* I and *Sal* I restriction site (designated by italics). The underlined nucleotide in the forward primer represents a nucleotide substitution which is silent in the P ORF but changes the start codon of the C ORF to threonine. The next start codon for the C ORF is more than 400 nucleotides downstream. Thus, only the P protein would be produced. The PCR product was then cloned into the *Nco* I-*Sal* I window of pTM-1 to yield a second plasmid, pTM(P no C).

15 The L ORF of HPIV3 was cloned into pTM-1 in three parts: the ends were derived from PCR products and the main body was a restriction fragment from p218(131). The upstream end of the L ORF was amplified using the primers 20 GCAAAGCGTGCCCGGGCCATGGACACTGAATCTAACAAATGGC (SEQ ID NO: 8) and GAAATTCCTTAATCGATTCTCTAGATTC (SEQ ID NO: 9). This yielded the 1,020-bp PCR product L1 in which positions 8625-9645 of the full-length genome were flanked by *Sma* I and *Nco* I sites on the upstream end and a *Cla* I site on the downstream end (all 25 three sites are italicized). The downstream end of the L ORF was amplified using the primers CCCATCAACTGTAACATACGTAAGAAAGAC (SEQ ID NO: 10) and GGTTAGGATATGTGACATTGTATTTATG (SEQ ID NO: 11). This yielded the 1,733-bp PCR product L2 in which 30 positions 13,645-15,378 of the full-length genome were flanked by a *SnaB* I and *Sal* I site (italicized). Plasmid p(131) was digested with *Cla* I and *Pst* I to yield the 4,487-bp fragment L middle containing positions 9,630-14,120 of the full-length genome. L1 and L middle were joined at the common *Cla* I site

and cloned into the *Sma I-Pst I* window of pBluescript to yield p(L1+L middle). The L2 fragment was then cloned into the *Pst I-Sal I* window of p(L1+L middle) to yield the complete L ORF flanked by *Nco I* and *Sal I*. This was then cloned into the *Nco I-Sal I* window of pTM-1 to yield pTM(L). The sequences of PCR-generated regions of pTM(N) (SEQ ID NO: 20), pTM(P) (SEQ ID NO: 21), and pTM(L) (SEQ ID NO: 22) were confirmed by the dideoxynucleotide sequencing method.

To increase the efficiency of T7 transcription, certain modifications were made to a cDNA construct encoding a negative-sense PIV minigenome, called PIV3-CAT(-) (Dimock and Collins, *J. Virol.* **67**: 2772-2778 (1993), incorporated herein by reference in its entirety). PIV3-CAT(-) includes the 3'-terminal 111 nucleotides and 5'-terminal 115 nucleotides of the HPIV3 genome fused to a negative-sense copy of the CAT ORF. This cDNA was designed to yield, upon linearization with *HgaI* and transcription with T7 RNA polymerase, a minigenome containing the exact correct ends of the HPIV3 genome. Two successive rounds of PCR, using mutagenic oligonucleotides which added successive extensions to the cDNA end, were used to replace the *HgaI* site with the hepatitis delta ribozyme (Perotta and Been, *Nature* **350**: 434-436 (1991), incorporated herein by reference in its entirety), such that self-cleavage generates the correct 3' HPIV3 genomic end. A T7 transcriptional termination signal was inserted immediately after the ribozyme (Fig. 3) to yield PIV3-CAT-delta.

PIV3-CAT-delta cDNA was modified by PCR mutagenesis to insert one, two or three G residues between the T7 promoter and the 5' end of the minigenome, using restriction sites flanking the trailer and T7 promoter. This modification yielded increased efficiency of T7 transcription. In preliminary experiments, the minigenome containing two G residues, called PIV3-CAT-GG, was the most active in the expression of CAT in the reconstituted transcription and replication system described below, and was used for all subsequent derivatives.

The PIV3-CAT-GG cDNA was further modified by overlapping PCR mutagenesis to introduce modifications

simultaneously at two sites, as follows. First, the sequence T₅CT, which contains tandem transcriptional termination motifs for the vaccinia virus early-stage RNA polymerase (T₅NT) (Yuen and Moss, Proc. Natl. Acad. Sci. U.S.A. **84**: 6417-6421 (1987), incorporated herein by reference in its entirety), was inserted into the positive-sense strand cDNA strand between the delta ribozyme and T7 transcriptional terminator (Fig. 1). This motif, and a second motif described herein below, were added to prevent promiscuous transcription of the CAT gene by the vaccinia virus early RNA polymerase. Second, the minigenome cDNA insert was modified at the junction between the N nontranslated region and CAT gene to contain (i) the insertion of a second vaccinia virus termination motif (T₅AT) into the positive-sense cDNA strand at positions 103 to 109 relative to the encoded minigenome, and (ii) the insertion of 0 to 6 G residues (negative-sense) at minigenome position 112 (Fig. 3). The overlapping PCR mutagenesis involved three sets of reactions (PCR 1, 2 and 3) performed as follows. PCR 1 was a set of seven parallel reactions [PCR1 (0) to (+6)] which used the PIV3-CAT-GG cDNA as a template and the following two mutagenic oligonucleotides as primers: the forward primer was: -¹¹¹GGGGTTATGCTACTGCAGGCTTTCTCCCTTAGCCATCCG-⁶² (SEQ ID NO: 12) and the reverse primer was: ¹²⁴CTCCATTCTAGA(N) TT**A**AAAAATTATAGAGTTCCC⁹⁰ (SEQ ID NO: 13). The bold sequence in the first oligonucleotide is the upstream tandem vaccinia terminator, and the bold sequence in the second oligonucleotide is the second terminator. This reaction amplified the ribozyme and adjacent leader region and inserted the mutations described above. PCR 2 was a single reaction that used the PIV3-CAT-GG cDNA as a template, and a forward primer that hybridized in plasmid sequence upstream of a unique *Ngo*MI site (Fig. 3), and a reverse primer complementary to the forward primer of reaction one. Thus, the products of PCR 1 and 2 overlapped at this latter sequence. The products of PCR 1 (0) to (+6) and PCR 2 were gel purified. The products of PCR1 (0) to (+6) were each mixed separately with an aliquot of PCR 2 product and amplified in a third reaction (PCR3 (0) to (+6)) which also

contained the forward primer of PCR 2 and the reverse primer of PCR 1. The products of PCR3 (0) to (+6) were digested with *NgoMI*, which cuts in plasmid-specific sequence, and *XbaI*, which cuts at the upstream end of the CAT gene (Fig. 3), and
5 cloned into the *NgoMI-XbaI* window of PIV3-CAT-GG. This resulted in a panel of cDNAs encoding minigenomes which were named according to the number of inserted G residues: PIV3-CAT 0 to PIV3-CAT +6. The structures of all DNA regions derived from PCR were confirmed by dideoxynucleotide sequencing.

10 C) Transfection

HEp-2 cells were grown to 90% confluence in 6 well plates. Each well of a six-well plate (1.5×10^6 cells) was transfected with 0.4 μ g pTM(P), 0.4 μ g pTM(N), 0.05 μ g pTM(L), and 0.4 μ g minigenome plasmid. The plasmids were added to 0.1 ml of OptiMEM (Life Technologies) and mixed with 0.1 ml of OptiMEM containing 12 μ l of LipofectACE (Life Technologies). After an incubation period of approximately 15 minutes at room temperature, 0.8 ml of OptiMEM 1 containing 2% calf serum and 1.5 $\times 10^7$ pfu of vTF7-3 was added to each well. The plates were incubated at 37°C for 12 hours after which the media was replaced with fresh OptiMEM 1 containing 2% fetal bovine serum. The cells were then incubated at 37°C for a total of 48 hours and harvested for RNA analysis and CAT assay. Each minigenome was represented in triplicate (3 wells) which was scraped into the medium and pooled.

20 25 D) CAT assay

An aliquot representing 3.33% (1.5×10^5 cells) of each pooled sample of harvested cells described above was removed for CAT assay. The aliquot was centrifuged at 1,000 rpm for 5 minutes and the supernatant discarded. The cell suspension was washed with 1 ml of 40 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl and resuspended in 50 μ l 0.25 M Tris, pH 7.5. Lysate was prepared by three cycles of freezing and thawing and clarified by centrifuging at 8,000 rpm for 5 minutes. 1 μ l of lysate was assayed for the ability to acetylate D-threo-[dichloroacetyl 1-¹⁴C]chloramphenicol (Amersham) using a conventional assay (Gorman et al., Mol. Cell. Biol. 2: 1044-1051 (1982), incorporated herein by

reference in its entirety). Acetylation was visualized by thin-layer chromatography and quantified by phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA).

5 E) RNA analysis

The remaining cell harvest of each pooled sample was divided into three equal parts for isolation of encapsidated RNA, total RNA, and mRNA. The three aliquots were centrifuged at 1,000 rpm for five minutes and the supernatants discarded. Two aliquots of cell suspension were resuspended in 50 μ l of RSB (10mM NaCl, 10mM Tris, pH 7.5, 1.5mM MgCl₂) containing 1% Triton X-100, 0.5% DOC. 50 μ l of 10 mM Tris 7.5, 1 mM CaCl₂, and 20 μ g (1mg/ml stock) of micrococcal nuclease was then added to one aliquot, and the other received the same mixture without micrococcal nuclease (Baker & Moyer, *J. Virol.* 62: 834-838 (1988), incorporated herein by reference in its entirety). The purpose of the micrococcal nuclease was to destroy nonencapsidated RNA, and the conditions used had been optimized in preliminary experiments. The mixtures were incubated at 30°C for 30 min and the RNA was isolated with Trizol (Life Technologies) according to the procedure of the supplier. The third aliquot of cell suspension was processed for RNA purification with Trizol and the purified RNA was separated by oligo(dT) cellulose chromatography into polyadenylated and nonpolyadenylated fractions (Grosfeld et al., *J. Virol.* 69: 5677-5686 (1995), incorporated herein by reference in its entirety). RNA samples were run on 1.5% agarose gels containing 0.44 M formaldehyde, transferred to nitrocellulose (Chomczynski, *Anal. Biochem.* 201: 134-139 (1992), incorporated herein by reference in its entirety), hybridized with strand specific riboprobes, and quantified by phosphoimager analysis.

35 EXAMPLE III

Construction and Expression of Modified PIV3 Minigenomes

In the present example, a panel of cDNAs was constructed to encode PIV3 minigenomes which differed in length by single nucleotide increments. Transcription and RNA

replication in this reconstituted system were the most efficient for the minigenome whose length was an even multiple of six. In this context, members of the *Paramyxovirus* and *Morbillivirus* genera typically abide by a "rule of six," i.e., genomes (or minigenomes) replicate efficiently only when their nucleotide length is a multiple of six (thought to be a requirement for precise spacing of nucleotide residues relative to encapsidating NP protein). However, the present findings illustrate that minigenomes whose lengths were one nucleotide greater than or less than an even multiple of six were surprisingly active, especially in RNA replication.

A panel of seven cDNAs was constructed to encode seven PIV3-CAT minigenomes, called PIV3-CAT 0 to +6, that differ in length by single-nucleotide increments (Fig. 3). Each minigenome is a short negative-sense analog of HPIV3 genomic RNA in which the viral genes had been deleted and replaced with a negative-sense copy of the CAT ORF. The CAT ORF is flanked by nontranslated segments of the N and L genes, the N GS and L GE transcription motifs, and the 3' leader and 5' trailer extragenic termini of genomic RNA. The 5' end of each PIV3-CAT minigenome is defined by the adjacent promoter for T7 RNA polymerase. This promoter was constructed to contribute an extension of two nonviral G residues to the 5' end of the encoded minigenome, as described above. The presence of additional G residues adjacent to the nontranscribed core of the T7 promoter improves its transcriptional efficiency, and preliminary work showed that presence of two G residues provided the highest levels of activity in the reconstituted system described below. These two G residues are not included in minigenome length calculations. The 3' minigenome end is created by self-cleavage by an abutting hepatitis delta virus ribozyme, which would generate the correct 3'-terminal nucleotide. The seven PIV3-CAT minigenomes differ by single-nucleotide increases in length due to the insertion of 0 to 6 G residues at the junction between the N nontranslated region and the CAT gene (Fig. 3), and range in length from 898 to 904 nucleotides. The PIV3-CAT +2 minigenome is the only one which

is an even multiple of six (it should be noted that each minigenome contained two additional nonviral G residues at the 5' end contributed by the T7 promoter, but it is assumed that these would be lost during intracellular HPIV3-mediated RNA replication).

Each PIV3-CAT cDNA was transfected into HEp-2 cells that had been infected with vTF7-3, a vaccinia virus recombinant that expresses T7 RNA polymerase. Plasmids encoding the N, P and L proteins under the control of the T7 promoter were transfected in parallel. The P cDNA had been modified by site-directed mutagenesis to eliminate the translational start site of the C ORF, as described above. Cells were harvested at 48 h post-infection. An aliquot of the cell suspension was processed for CAT enzyme assay (Fig. 4). The remaining cells were divided into three equal aliquots and processed for RNA analysis as described below.

The minigenome cDNA was further modified to contain two tandem vaccinia virus early-gene transcription termination motifs (T₇NT) in the positive-sense plasmid strand upstream of the PIV3-CAT insert, and a third one (T₅AT) in the same strand immediately upstream of the CAT ORF (Fig. 3). These were designed to minimize promiscuous transcription of the CAT ORF by vaccinia virus polymerase (Grosfeld et al. (1995), *supra*). CAT expression was reproducibly detected when each of PIV3-CAT minigenomes was complemented by the N, P and L plasmids (Fig. 4), and detection of CAT was dependent on all three PIV3 proteins. However, expression was much higher for PIV3-CAT+2, which has a nucleotide length that is an even multiple of six. Preferred ratios and amounts of the minigenome and support plasmids were determined based on CAT enzyme expression.

EXAMPLE IV

Synthesis of Positive-Sense RNAs by PIV Minigenomes

Transcription and replication of PIV minigenomes was confirmed by detection of RNA products of both processes. As described in the foregoing Example, three equal aliquots of cell suspension were taken for RNA analysis. One aliquot was

used for oligo(dT) analysis, as described below. The other two aliquots were lysed with detergent and incubated with micrococcal nuclease or mock-treated. RNA was then isolated, separated by electrophoresis on formaldehyde agarose gels, transferred to nitrocellulose, and analyzed by hybridization with negative-sense CAT riboprobe. RNA from micrococcal-treated and mock-treated lysates are shown in Fig. 5A upper and lower panels, respectively.

Analysis of RNA from mock-treated lysates showed that complementation of each minigenome with the N, P and L plasmids resulted in the synthesis of a band of RNA which was very similar in size to a marker consisting of RNA expressed by the 931-nucleotide RSV-CAT C2 minigenome (Grosfeld et al. (1995), supra). Phosphorimager analysis is shown in Fig. 5B. Little or no RNA was detected when the N or L plasmids were omitted, confirming that these RNAs are products of the reconstituted PIV3 polymerase.

Each PIV3-CAT minigenome is expected to encode two positive-sense RNAs, namely the mini-antigenome and the subgenomic CAT mRNA. Each mini-antigenome is expected to be the exact complement of its minigenome, which was 898 to 904 nucleotides in length. The predicted subgenomic mRNA is defined by the GS and GE signals, and is expected to be 804 nucleotides in length and contain a polyA tail of 100 to 200 nucleotides.

Detection of a single gel band of positive-sense RNA in Fig. 5A (lower panel) suggested that the antigenome and mRNA were not resolved by gel electrophoresis. Accordingly, treatment with micrococcal nuclease was used to identify antigenome RNA, since the antigenome (and genome) but not mRNA would be encapsidated and resistant to digestion. The use of micrococcal nuclease for this purpose is well established (Baker & Moyer (1988), supra), and the conditions selected were verified with RSV minireplicons and shown to completely degrade mRNA contained in the HEp-2 cell lysates. Residual RNA was purified and analyzed by Northern blot analysis with negative-sense riboprobe (Fig. 5A, upper panel) and quantitated by phosphorimager (Fig. 5B; note that in this

analysis the micrococcal-treated and untreated RNA amounts were normalized separately). These investigations revealed the presence of a population of protected RNA corresponding to the positive-sense encapsidated mini-antigenome. Among 5 several experiments, this protected RNA accounted for approximately 3 to 15% of the positive-sense RNA.

For both the total and the micrococcal-resistant RNA, accumulation was greatest in the case of the +2 minigenome, which is 900 nucleotides in length and thus a 10 multiple of six. However, substantial amounts of RNA also accumulated in the case of the minigenomes which did not exhibit a length corresponding to a multiple of six nucleotides, in particular minigenomes +1 and +3 which were one nucleotide longer or shorter than the +2 minigenome. In 15 fact, the amount of encapsidated antigenome produced by the +1 and +3 minigenomes was 85% and 72% that of the +2 minigenome (Fig. 5B). Even the least efficient minigenome, the +5 minigenome, was 20% as active as the +2 minigenome as determined by measurement of accumulated encapsidated RNA. In 20 the case of measurements to detect total positive-sense RNA, the +1 and +3 minigenomes produced 52% and 45% as much total RNA as the +2 minigenome.

To confirm the presence of subgenomic mRNA, the final aliquot of harvested cell suspension was processed for 25 RNA purification. The RNA was then subjected to oligo(dT) chromatography. RNAs which failed to bind, and those which bound and were eluted in low salt buffer, were analyzed by Northern blot hybridization (Fig. 6A) and phosphorimager (Fig. 6B; note that in this case the bound and unbound are 30 normalized together relative to the bound RNA of the +2 minigenome). These assays showed that approximately 64% of positive-sense RNA was polyadenylated, as expected for 35 subgenomic mRNA. The accumulation of mRNA was greatest for the +2 minigenome. However, substantial amounts of mRNA also were observed for the other minigenomes. The amount of mRNA synthesized by the +1 and +3 minigenomes was 30% and 20% respectively compared to that synthesized by the +2

minigenome, and was approximately 13% for the least active minigenomes.

EXAMPLE V

5 Synthesis of Negative Sense RNA by PIV Minigenomes

The various PIV3-CAT minigenomes described in the foregoing examples directed synthesis of mRNA and positive-sense encapsidated mini-antigenome, the latter representing the first step in RNA replication. The second step in RNA replication involves synthesis of encapsidated progeny minigenome from the mini-antigenome product. To evaluate this latter process, the samples of RNA from mock-treated and nuclease-treated lysates described in the preceding Example were analyzed by Northern blot hybridization with positive-sense CAT riboprobe (Fig. 7A) and quantitated by phosphorimager (Fig. 7B).

Analysis of RNA from mock-treated lysates (Fig. 7A, lower panel) showed that considerable amounts of minigenome accumulated intracellularly in all samples, including negative controls in which the N or L support plasmid was omitted. The analyses described in Figs. 5A-B and 6A-B showed that the synthesis of positive-sense RNA was insignificant under these conditions. Therefore, the minigenome observed in the absence of N or L could not be the product of RNA replication mediated by the reconstituted HPIV3 polymerase, and instead must be the product of T7 transcription of transfected plasmid.

Minigenome produced by the reconstituted HPIV3 polymerase is expected to be encapsidated, whereas much of the minigenome produced by T7 RNA polymerase is expected to be unencapsidated. Therefore, RNA from the same micrococcal nuclease-treated samples described for Figs. 5A-B were used to prepare a second blot, which was hybridized with positive-sense CAT riboprobe (Fig. 7A, upper panel). This showed that all minigenome RNA accumulated in the absence of the N protein was degraded (Fig. 7A, upper panel, lane 1), as expected. Essentially all of the minigenome which accumulated in the absence of L was also sensitive to degradation (Fig.

7A, upper panel, lane 2). Plasmid-derived minigenome synthesized in the absence of L, and in the presence of N and P alone, did not appear to occur efficiently.

When the complete set of three support plasmids was present, significant amounts of micrococcal nuclease-resistant minigenome RNA accumulated for each of the minigenomes (Fig. 5 7A, upper panel). As was the case with the positive-sense RNAs, the greatest amount of progeny minigenome was observed 10 with the +2 minigenome. The +1 and +3 minigenomes were next in abundance, with levels of genomic RNA that were 67% and 42% of that of the +2 minigenome.

The foregoing examples demonstrate that the HPIV3 N, P and L proteins were necessary and sufficient for efficient transcription and RNA replication. The very robust nature of transcription and RNA replication mediated by the 15 reconstituted PIV3 polymerase confirmed the functionality of the encoded proteins. It is further expected that inclusion of additional viral proteins within the expression system will augment or modify these processes. Coexpression of PIV C, D and potentially V, within the compositions and methods of the invention will be useful, e.g., to augment and/or modify RNA 20 replication. For this purpose, plasmids will be constructed and assayed according to the foregoing methods to achieve coexpression of one or more of these elements to determine 25 their effects on PIV transcription and RNA replication, as well as on PIV phenotype in suitable infection models.

EXAMPLE VI

30 Construction of Infectious, Recombinant PIV from cDNA

The following examples describe production of infectious, recombinant PIV (rPIV) by intracellular 35 co-expression of four plasmid-borne cDNAs. These cDNAs separately encode a complete HPIV3 genome and the HPIV3 nucleocapsid protein N, the phosphoprotein P, and the polymerase protein L.

A) Viruses and cells

Modified vaccinia strain Ankara (MVA), the vaccinia virus recombinant that expresses bacteriophage T7 RNA polymerase, was provided according to Wyatt et al., Virol. 210:202-205, 1995, incorporated herein by reference in its entirety. HEp-2 monolayer cultures were maintained at 37° C in 5% CO₂ with OptiMEM 1 (Life Technologies) supplemented with 2% FBS, 50µg/ml gentamicin sulfate and 2mM glutamine. The JS wt strain of HPIV3 and its attenuated ts derivative, JS cp45, were propagated in LLC-MK2 cells as described by Hall et al., Virus Res. 22: 173-184, (1992), incorporated herein by reference in its entirety.

B) cDNAs

The full cDNA clone designated p218(131) (Fig. 1, SEQ ID NO: 1) encoding a complete genomic sequence of HPIV3 was constructed as described above. A second cDNA clone designated p3/7(131) (SEQ ID NO: 14) (deposited under the terms of the Budapest Treaty with the ATCC, and granted the designation 97990) was constructed to encode a complete antigenomic sequence of HPIV3. p3/7(131) differs from p218(131) in that the positions of the T7 promoter and the ribozyme/T7 terminator relative to the HPIV3 cDNA insert have been interchanged. Thus, the first nucleotide synthesized in p3/7(131) is the 5' end of the antigenome, namely the positive-sense complement of genome position 1, and the 3' antigenome end defined by ribozyme cleavage is the complement of genome position 15462. A third clone designated p3/7(131) 2G (SEQ ID NO: 15) (deposited under the terms of the Budapest Treaty with the ATCC, and granted the designation 97989) was also constructed identical to p3/7(131), except that two G residues were inserted between the 5' end of the antigenome and the T7 promoter.

For construction of p3/7(131) 2G and p3/7(131), the two plasmids p(A*A'CC'D*E) and p(E*FF'GHIJKL*) were modified and joined to encode the complete positive-sense antigenome of HPIV3. First, the T7 terminator and delta ribozyme abutting the 3' end of HPIV3 in p(A*A'CC'D*E) were replaced by a T7 promoter using PCR (see Fig. 8). The positive-sense primer

5'-GCCCGTCGAC**GCGTAATACGACTCACTATA**

GGACAAACAAGAG-3' (SEQ ID NO: 18) placed the T7 promoter (bold) adjacent to the 3' leader of HPIV3 (HPIV3 sequence underlined) with 2 G residues inserted between these two elements to improve transcription.

5 A unique *Mlu* I site (italicized) was placed upstream of the T7 promoter for cloning purposes (Fig. 8). The negative-sense primer 5'-¹²²⁴CGGCATCACGTGCTAC¹²⁰⁹- 3' (SEQ ID NO: 19) spanned nt 1209-1224 of the HPIV3 N gene and included a unique *Pml* I site (italicized) present in the natural HPIV3 sequence. Both 10 the PCR product and the parent template p218(A*A'CC'D*E) were digested with *Mlu* I and *Pml* I and the PCR product was then cloned into the *Mlu* I-*Pml* I window. A second PCR reaction was done using the same negative-sense primer and a positive-sense primer without 2G residues inserted between the T7 promoter and 3' end of HPIV3. These plasmids were designated p(Left + 2G) and p(Left +). The construction of p(Left + 2G) is 15 illustrated in Fig. 8, the construction of p(Left +) followed the same strategy.

20 Plasmid p(E*FF'GHIJKL*) was modified by PCR to place the delta ribozyme and T7 terminator adjacent to the 5' end of HPIV3 (Fig. 9). The positive-sense primer:

5'-GGATTGCGCGC¹⁴⁸¹³AATTAAATCATCTGG¹⁴⁸²⁸-3' (SEQ ID NO: 16) introduced a *BssH* II site (italicized) just upstream of a unique *Swa* I site (italicized, underlined) in the L gene of 25 HPIV3 (HPIV3 specific sequence is underlined). The negative-sense primer:

5'-CCCAGGTGGACCGCGAGGAGGTGGAGATGCCATGCCAGCCC¹⁵⁴⁶²ACCA
AAACAAAGAGAAGAACTCTGTTGG¹⁵⁴³⁵- 3' (SEQ ID NO: 17) placed a portion of the delta ribozyme (bold) which included a 30 naturally-occurring unique *Rsr* II site (italicized) adjacent to the 5' end of the HPIV3 genome (negative-sense underlined). This PCR product was digested with *BssH* II and *Rsr* II and cloned into the *BssH* II-*Rsr* II window of plasmid p3/7 (Fig. 9). Plasmid p3/7 is identical to p218 except that the delta 35 ribozyme and T7 terminator are in the reverse orientation. The p3/7 plasmid containing the above-mentioned insertion was designated pPIV3-3/7 and contained the complete delta ribozyme and T7 terminator adjacent to 5' terminal 651 nucleotides of

HPIV3. The *SwaI* and *NgoM*I fragment of plasmid pPIV3-3/7 was then isolated and cloned into the *Swal-NgoM* I window of p(E*FF'GHIJKL*). The resulting plasmid, designated p(Right +) placed the complete delta ribozyme and T7 terminator adjacent 5 to the 5' end of HPIV3 (see Fig. 9). The *Xho* I -*NgoM* I fragment of p(Right +) was cloned into the *Xho* I -*NgoM* I window of p(Left +) and p(Left + 2G) resulting in plasmids p3/7(131) (SEQ ID NO: 14) and p3/7(131 2G) (SEQ ID NO: 15), respectively (Fig. 10). These each encode the complete 10 positive-sense analog of HPIV3 antigenomic RNA, with the latter cDNA containing two G residues adjacent to the T7 promoter for improved transcriptional efficiency.

C) Transfection

HEp-2 cells were grown to 90% confluence in six well plates. Each well of a six-well plate (1.5×10^6 cells) was transfected with the three previously-described support plasmids, $0.4\mu\text{g}$ pTM(P), $0.4\mu\text{g}$ pTM(N), $0.05\mu\text{g}$ pTM(L), together with $5\mu\text{g}$ of full-length genomic or antigenomic HPIV3 cDNA. The plasmids were added to 0.2 ml of OptiMEM 1 (Life Technologies) containing $12\mu\text{l}$ of LipofectACE (Life Technologies). After an incubation period of approximately 15 minutes at room temperature, 0.8 ml of OptiMEM 1 containing 2% fetal bovine serum and 1.5×10^7 pfu of MVA-T7 was added to each well. The cultures were incubated at 32°C for 12 hours, 20 after which the media was replaced with fresh OptiMEM 1 containing 2% fetal bovine serum. The cultures were incubated at 32°C for three additional days then harvested and passaged (referred to as passage 1) onto fresh HEp-2 monolayers. These 25 passage 1 cultures were incubated at 32°C for five days, and virus present in the culture was harvested, passaged once in LLC-MK2 cultures, and characterized by hemagglutination-inhibition (HAI) as described (van Wyke Coelingh et al., *Virol.* 143: 569-582, (1985) (incorporated herein by reference in its entirety) to determine if it 30 possessed the monoclonal antibody resistant mutation (MARM) that marked virus recovered from cDNA.

D) Sequencing of Recombinant Virus

The presence of nucleotide sequence markers in the HN and L genes of recombinant PIV was determined by RT-PCR of RNA isolated from recovered virions. 1 ml of rPIV (1 X 5 10^5 pfu/ml, passage level 2) was precipitated with 200 μ l 25% polyethylene glycol by incubation on ice for one hour and centrifuging at 12,000g for 15 minutes. The RNA was purified with TRIZol reagent (Life Technologies) following the manufacturer's recommended procedure. RT-PCR was performed 10 with the Superscript kit (Life Technologies) following the manufacturer's recommended protocol. Control reactions were identical except that reverse transcriptase was omitted from the reaction to confirm that the PCR products were derived solely from virus RNA and not from possible contaminating cDNA plasmids. Four primer pairs were used to generate PCR 15 products from nt 7334-8715, 9364-10854, 10939-15392, and 13623-15392. The resultant PCR products were then sequenced using cycle dideoxynucleotide sequence analysis (New England Biolabs, Beverly, MA).

20 EXAMPLE VIIRecovery of Recombinant Virus From cDNA Encoding
Negative-Sense Genomic RNA

25 Plasmid p218(131) and the three support plasmids pTM(N), pTM(P), and pTM(L) were transfected into HEp-2 cells with MVA expressing T7 RNA polymerase. A control group consisting of pTM(N), pTM(P), pTM(L), and MVA was cotransfected into HEp-2 cells without p218(131). On day 30 four, the transfection was harvested, passaged onto fresh HEp-2 cell monolayers for five days, and passaged again for 5 days in LLC-MK2 cultures (passage 2). Virus present in the passage 2 harvest was further characterized by HAI. Cultures from the transfection group which received the three support 35 plasmids without the full-length genomic clone p218(131) did not yield HPIV3. The rPIV recovered virus was confirmed to be HPIV3 since it reacted in the HAI assay with the mAbs 77/5, 101/1, and 454/11 which are specific for HPIV3 (Table 1). It

was presumptively identified as being cDNA-derived because it failed to react with mAbs 170/7 and 423/6, consistent with the MARM mutation which had been introduced into the cDNA.

TABLE 1

rJS-NS Contains the MARM Mutation Introduced into the p218(131) Full-Length Negative Sense cDNA

	Hemagglutination-inhibiting	Antibody Titer	(Reciprocal) of Indicated mAb	
Virus	77/5 ¹	101/11	454/11 ¹	170/1 ²
JS wt ³	800	6400	6400	25,600
rJS-NS ⁴	3200	25,600	6400	≤ 25

¹ mAb 77/5 recognizes antibody epitope IIB, mAbs 101/1 and 454/11 recognize antibody epitope IIIA of HN glycoprotein, all of which were not altered in p218(131).

² Mabs 170/7 and 423/6 which both recognize antibody epitope I of JSwt, fail to recognize rJS due to the MARM mutation at this site.

³ Biologically derived wild type HPIV3 JS.

⁴ Recombinant JS virus derived from negative-sense genomic cDNA.

To confirm that rPIV was indeed recovered from cDNA, it was analyzed in parallel with wild-type JS strain HPIV3 by RT-PCR using four primer pairs flanking the seven inserted marker mutations. Each PCR product obtained was dependent upon the inclusion of RT, indicating that each was derived from RNA and not from contaminating cDNA. Cycle-sequencing of the four PCR products confirmed that the sequence of the rPIV contained each of the seven markers, sequencing data showing three of the markers is illustrated in Figure 11. The sequence differences between rPIV and JS wt in the HN gene, including nt 7903, 7913, and 7915 are readily apparent. Similar sequence analyses were carried out for the other four markers at nt positions 7593, 10355, 11333, and 15248, and the rPIV possessed each mutation.

These results demonstrate successful recovery of infectious rPIV from cDNA encoding a negative-sense genomic RNA. This differs from most published reports for recovery of nonsegmented negative strand RNA viruses, in which the cDNA used for virus recovery had been designed to encode positive-sense antigenomic RNA (Baron and Barrett, supra, 1997; Collins et al., supra, 1995; Conzelmann, supra, 1996; Garcin et al., supra, 1995; Lawson et al., supra, 1995; Radecke et al., supra, 1995; Whelan et al., supra, 1995). The recovery of infectious virus from a cDNA encoding genomic RNA had previously been reported only in the case of Sendai virus, and the efficiency of recovery was much lower than for cDNA encoding antigenomic RNA (Kato et al., 1996). In most other studies, the recovery of virus was achieved with antigenomic cDNA but not with genomic cDNA (Lawson et al., 1995; Whelan et al., 1995). A number of potential problems have been noted which may explain these refractory results, including possible annealment of cDNA-encoded genomic RNA with mRNA produced by the support plasmids; resulting in inactive hybrids (Conzelmann, supra, 1996; Lawson et al., supra, 1995). It has also been noted that T7 RNA polymerase appears to terminate preferentially at the gene junctions of genomic RNA, perhaps because the oligo U tract of the GE signal resembles

the natural signal for transcription termination by T7 RNA polymerase (Whelan et al., supra, 1995).

5

EXAMPLE VII

Recovery of Recombinant Virus From cDNA Encoding Positive-Sense Antigenomic RNA

As described in more detail above, p3/7(131) and
10 p3/7(131)2G were constructed to encode a positive-sense,
antigenome that give rise to recombinant PIV. Plasmid
p3/7(131)2G is identical to p3/7(131) but for the addition of
two G residues between the T7 promoter and the first
nucleotide of the antigenome. The addition of two G residues
15 between the T7 promoter and the first HPIV3 nucleotide
p3/7(131)2G is based on the preceding examples demonstrating
that the presence of the two added G residues (as opposed to
0, 1 or 3 added G residues) yielded substantially increased
levels of minireplicon replication.

The two antigenome cDNAs [p3/7(131) and p3/7(131)2G]
20 were transfected separately into cells together with the N, P
and L support plasmids, and were infected simultaneously with
the MVA-T7 recombinant virus using the same procedure
described above for p218(131). Infectious virus from each
25 antigenomic cDNA was recovered and was presumptively
identified as being cDNA-derived by its inability to react
with mAbs 423/6 and 170/7.

The efficiency of virus recovery was evaluated for
each of the antigenome cDNAs versus the genome cDNA p218(131).
30 Twelve transfection reactions using the negative-sense genome
cDNA p218(131) (SEQ ID NO: 1) were conducted in parallel with
twelve transfections using the positive-sense antigenome cDNA
p3/7(131)2G (SEQ ID NO: 15) to compare efficiency of virus
recovery from the two cDNAs. One ml of the transfection
35 harvest from each well was titered on LLC-MK2 cells and the
remaining 2mls were passaged (passage 1) onto fresh LLC-MK2
cells. At day five, passage 1 was harvested and titered as
described above. Recombinant virus was recovered from 12/12

wells transfected with p3/7(131) 2G but from only 4/12 wells
transfected with p218(131). The mean titer of virus present
in culture of the passaged virus from the positive-sense
antigenome was $10^{5.0}$, nearly ten-fold higher than that from the
negative-sense genome, which was $10^{4.1}$. However, with one
5 additional amplification the titers became equivalent.

TABLE 2

Comparison of the Efficiency of Recovery of Recombinant Virus from cDNAs Encoding Genomic or Antigenomic RNA, the latter with or without 2G residues adds to the T7 promoter.

Experiment#	Transfected cDNA	Sense of Encoded RNA	Rescue Efficiency ¹	Mean Titer (\log_{10} pfu/ml) of Recovered Virus ²
#1	p3/7(131) 2G ³	Antigenomic	12/12 (100%)	5.0 ± 0.27
	p218(131)	Genomic	4/12 (33%)	4.1 ± 0.27
#2	p3/7(131) 2G ³	Antigenomic	6/6 (100%)	6.6 ± 0.18
	p3/7(131)	Antigenomic	12/12 (100%)	5.3 ± 0.12
	p218(131)	Genomic	5/5 (100%)	4.9 ± 0.19

1 Number of transfection cultures yielding rJS/number tested.

2 The mean Titer ± standard error was determined following one passage of transfection harvest for five days in LLC-MK2 cells.

3. Contains 2 G residues between the T7 promoter and the 5' end of the antigenome.

The efficiency of recovery of recombinant virus from the three full-length plasmids encoding the genomic or antigenomic HPIV3 RNAs was next studied, (i) to determine whether genomic or antigenomic cDNA is more efficient at generating recombinant virus and (ii) to determine the effect of two extra 5' terminal G residues on the yield of recombinant viruses (Table 2.) Unfortunately, it was not possible to directly titer the transfection harvest by plaque titration because residual MVA-T7 interfered with plaque formation of rJS on LLC-MK2 monolayer cultures. Therefore, we first compared the efficiency of recovery of rJS from multiple independent transfections and, as seen in other experiments, found that rJS was more frequently recovered from cultures transfected with p3/7(131)2G than from cultures transfected with p218(131). Since each transfection harvest, including those with p218(131) yielded rJS in other experiments, it was not possible to use this method of analysis to compare the relative efficiency of generating recombinant virus. We therefore estimated the relative quantity of virus present in the transfection harvest by quantitating the amount of virus following one passage of the transfection harvest (Table 2). Since each plasmid was designed to yield an identical virus (rJS), differences in titers of the passaged virus were considered a reflection of differences in the titer of virus present in transfection harvest. In the above experiments, the construct with two 5' terminal G residues was the most efficient in generating rJS. This indicated that the two added residues indeed increased efficiency of recovery, but the mechanism for this small increment in efficiency was not defined. These findings indicate a small, but measurable, advantage of using p3/7(131)2G as substrate for future experiments designed to introduce mutations into the HPIV3 cDNA. The small advantage in recovery of recombinant virus from p3/7(131)2G might be especially needed to recover viruses that have attenuating mutations that restrict replication in vitro. Although VSV has been recovered from antigenomic cDNA which included three G residues at the 5'-terminal end, the efficiency of recovery of virus from this construct was not

compared with recovery from the same construct lacking the extra residues (Whelan et al., Proc. Natl. Acad. Sci. USA 92:8388-8392 (1995), incorporated herein by reference). In contrast to the experience with Sendai virus and measles virus, it is clear that extra-viral G residues at the 5'-terminal end of the antigenome are not deleterious to recovery of recombinant virus, and, in fact, appear to be advantageous [Garcin et al., supra, 1995; Radecke et al., supra, 1995; Kato et al., 1996). The genomic and antigenomic plasmids without two 5'-terminal guanine residues appeared equally efficient in generating recombinant virus. This is in contrast to previous reports which have found genomic cDNA to be less efficient than antigenomic cDNA in generating recombinant virus (Whelan, 1995; Kato et al., 1996).

The efficiency of recovery of virus from p3/7(131) (SEQ ID NO: 14) was compared to that of p3/7(131)2G (SEQ ID NO: 15 and p218(131) (SEQ ID NO: 1) in a similar experiment as above. Recovery from p3/7(131) was comparable to that of p217(131). Recovery was more consistent and efficient with both antigenome cDNAs containing the extra Gs (Table 2).

EXAMPLE VII

Efficiency of plaque formation of rPIV at permissive and restrictive temperatures

The level of temperature sensitivity of replication *in vitro* of control viruses and rPIV derived from both p218(131) and p3/7(131)2G was determined at 32°C, 37°C, 39°C, and 40°C in LLC-MK2 monolayer cultures as previously described (Hall et al., Virus Res. 22: 173-184, (1992), incorporated herein by reference in its entirety), with the following modifications. Viruses were inoculated onto LLC-MK2 monolayers on 24-well plates in serial 10-fold dilutions allowed to adsorb for one hour at room temperature. The cultures were then overlaid with 1ml of L-15 supplemented with 2mM glutamine and 0.8% methylcellulose and the plates were then incubated for 5 days at the indicated temperature. The methylcellulose overlay was removed and the monolayer fixed

with 80% methanol at 4°C for 1h. The viral plaques present in the monolayer were scored using a mixture of two HPIV3-specific anti-HN murine mAbs 101/1 and 66/4 as ascites fluid used at a dilution of 1:500, using an immunoperoxidase method of staining specific for murine antibodies as previously described (Murphy et al., Vaccine 8: 497-502 (1990), incorporated herein by reference in its entirety).

Recombinant virus derived from either positive or negative-sense cDNA were characterized by plaque assay at 32°C, 37°C, 39°C, and 40°C to determine if they were phenotypically similar to JS wt virus. Both positive and negative-sense rPIV were comparable to the JS wt virus in their level of replication at elevated temperatures of 39°C and 40°C (Table 3). This is in contrast to the *ts* mutant JS *cp45* which exhibits a 30-fold reduction in titer at 37°C and fails to produce plaques at 39°C or 40°C.

TABLE 3

The rJS Resembles its Biologically Derived Parent JS Wild-Type Virus in the Level of Replication at Restrictive Temperature (39°C - 40°C)

	Virus	Virus Titer (log 10 pfu/ml)			
		32°C	37°C	39°C	40°C
	rJS-PS ¹	6.1	6.1	6.1	6.6
	rJS-NS ²	6.9	7.1	7.1	7.0
	JS <i>cp45</i> ³	6.3	4.3	<0.7	<0.7
	JS wt	6.5	6.8	6.6	6.7

¹ Recombinant virus derived from the antigenomic-sense clone p3/7(131)2G

² Recombinant virus derived from the genomic-sense clone p218(131)

³ JS*cp45* is a temperature sensitive mutant derived from JS wt.

The sequence of JS *cp45* has been fully determined (Stokes et al., *supra*, 1993) and mutations have been identified in the leader, N, P, M, F, HN, and L genes. However, it is unknown which mutation(s) are responsible for the *ca*, *att*, or *ts* phenotypes. Because exemplary rPIV of the invention demonstrate the *ts** phenotype like the JS wt parent, *cp45* mutations among other mutations known or yet to be discovered for PIV can be introduced, alone or in combination, into the full-length cDNA to pinpoint the effects of individual mutations or combinations thereof, e.g., by evaluating replication of the recombinant virus incorporating the mutation(s) of interest at elevated temperatures. The mutation(s) thus identified can be incorporated into effective, attenuated vaccine agents as described in the Examples below. These and other mutations incorporated into recombinant PIV can be further optimized by, e.g., mutagenesis to create two or more nucleotide substitutions per codon to render a recombinant virus that is more genetically stable than a biologically derived mutant strain.

EXAMPLE VIII

Replication of rPIV in Hamsters

Thirty-six 16-week-old golden Syrian hamsters were divided into four groups of nine and inoculated intranasally with 0.1ml containing $10^{5.5}$ pfu of either rPIV recovered from negative-sense cDNA, rPIV recovered from positive-sense cDNA, JS *cp45*, or JS wt virus. On day 4, the hamsters were sacrificed and the lungs and nasal turbinates harvested. The lungs were homogenized in a 20% w/v L-15 suspension containing 2.5 μ g/ml amphotericin B (Quality Biologicals, Gaithersburg, MD), 200 μ g/ml pipericillin (Lederle Laboratories, Pearl River, NY), and 50 μ g/ml gentamicin (Quality Biologicals). The nasal turbinates similarly were homogenized in a 10% w/v L-15 suspension. After homogenization, the samples were aliquoted and rapidly frozen in a dry ice-ethanol bath. Virus present in the samples were titered at a later date in 96 well plates

of LLC-MK2 cells at 32°C scoring CPE at five and seven days after inoculation. The mean \log_{10} TCID₅₀/gm was calculated for each group of nine hamsters.

Table 4 illustrates that rPIV recovered from negative-sense cDNA, rPIV recovered from positive-sense cDNA replicate to substantially the same level as the JS wt in the upper and lower respiratory tract of hamsters. This is in contrast to the JS cp45 virus, which is attenuated at each site.

TABLE 4

The rJS Resembles its Biologically Derived Parent JS wt Virus in the Level of Replication in the Upper and Lower Respiratory Tract of Hamsters.

	Virus	Mean Virus Titer (\log_{10} TCID ₅₀ /g) ⁵	
		Nasal Turbinates	Lungs
	rJS-PS ¹	6.6 ± 0.2	4.1 ± 0.3
	rJS-NS ²	6.4 ± 0.1	4.2 ± 0.2
	JScp45 ³	4.2 ± 0.2	≤ 1.4 ± 0.0
	JS wt ⁴	6.3 ± 0.2	4.6 ± 0.3

¹ Recombinant virus recovered using p3/7(131)2G encoding the positive-sense HPIV3 antigenome.

² Recombinant virus recovered using p218(131) encoding the negative-sense HPIV3 genome.

³ Biologically derived ts mutant.

⁴ Biologically derived parent virus.

⁵ Mean titers ± standard errors for nine hamsters per group.

Thus, exemplary rPIVs of the invention can retain the replicative capacity in hamsters exhibited by the biologically derived JS wt parent strain, whereby mutations such as those present in the JS cp45 candidate vaccine that restrict replication in hamsters and other hosts, including

non-human primates and humans, can be identified and incorporated within modified rPIV strains of the invention, as described in the Examples below.

5

EXAMPLE IX

Identification of Amino Acid Substitutions in HPIV3 Specifying Attenuated Phenotypes, and Incorporation of Attenuating Mutations into Infections, Attenuated PIV Clones

10 The ability to generate infectious PIV from cDNA facilitates development of live-attenuated parainfluenza virus vaccines. More specifically, by using the methods and tools disclosed herein the genetic basis of attenuation of PIV candidate vaccines can be readily determined, and infectious PIV vaccines produced from cDNA can be designed to achieve a finely calibrated level of attenuation and immunogenicity.

15 In addition, the tools and methods of the invention provide for vaccine development for all three human parainfluenza viruses, HPIV1, HPIV2 and HPIV3 that are most important in human disease. For example, to produce and select effective HPIV3 vaccine agents within the invention, mutations associated with desired phenotypes of biologically deriving HPIV3 candidate vaccines or the attenuated BPIV3 virus, e.g. attenuating mutations, can be identified and 20 incorporated into rPIV. Applying these methods, attenuating mutations from a large menu of candidate mutations are selected and combined to generate rPIV having a desired balance between attenuation and immunogenicity, and which retain the attenuation phenotype following replication in 25 humans.

30 In the present example, the genetic bases of temperature-sensitive (*ts*) and *in vivo* attenuation (*att*) phenotypes of the PIV3 JS cp45 live-attenuated virus are described. Seven exemplary recombinant PIV3 viruses (three single-, three double-, and one triple-lesioned virus) were 35 recovered from full-length antigenomic cDNA and analyzed for their *ts* and *att* phenotypes. These recombinants bore one or more amino acid substitution mutations present in the L gene

of JS *cp45* (alternatively referred to herein as *cp45*), adopted within a cDNA clone of the JS wt parent. These three exemplary, biologically derived mutations are all present in a representative strain of JS *cp45* grown in Vero cells,
5 designated JS *cp45* Vero, deposited on August 21, 1997 under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., and granted the accession number ATCC VR 2588.

10 Analyses of exemplary PIV recombinants, presented below, demonstrate that each of the three exemplary mutations in L (Tyr₉₄₂ to His, Leu₉₉₂ to Phe, and Thr₁₅₅₈ to Ile) contribute to the *ts* and *att* phenotypes of *cp45* and are useful for generating of recombinant vaccine virus.
15

Viruses and cells.

The PIV3 JS wt and *cp45* viruses were grown in LLC-MK2 cells as described previously (Hall et al., *Virus Res.* 22:173-184 (1992), incorporated herein by reference). The vTF7-3 recombinant vaccinia virus is described in Fuerst et al., *Virology* 225: 419-422 (1996) and the modified vaccinia virus Ankara (MVA) which expresses the T7 polymerase is described in Wyatt et al., *Virology* 210:202-205 (1995) (each incorporated herein by reference). HEp-2 (ATCC CCL 23) and
20 LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM (Life Technologies) supplemented with 2% FBS and gentamicin sulfate (50ug/mL).
25

Construction of point mutations in the L gene of PIV3.

30 pUC19 was modified to accept a fragment of the JS wt PIV3 L gene in order to introduce point mutations into the L gene by site-directed mutagenesis. First, a unique *Nhe* I restriction site was introduced into pUC19 by ligating a pair of complementary oligonucleotides (5' GATCGATGCTAGCCC 3' (SEQ ID NO: 23) and 5' GATCGGGCTAGCATC 3' (SEQ ID NO: 24)) containing an *Nhe* I restriction site into the *Hind* III site of pUC19 to create pUC19 (N). The *Sph* I (PIV3 nt 11317) to *Nhe* I (PIV3 nt 14087) fragment of pTM(L), which includes the

positions where the three coding changes in cp45 occur and which can be directly introduced into the full-length PIV3 cDNA (see below), was cloned into the *Sph* I and *Nhe* I site of pUC19 (N) to create pUCL(N-S). Point mutations were
5 introduced into pUCL(N-S) using mutagenic oligonucleotides with the Transformer mutagenesis kit (Clontech, Palo Alto, CA) for the purpose of (i) creating exemplary amino acid substitutions at L protein positions 942, 992, and 1558, individually and in combination, and (ii) ablating one
10 specific naturally-occurring restriction enzyme site proximal to each codon substitution as a marker [See Table 5].

TABLE 5: Nucleotide substitutions introduced into rPIV3 that encode cp45L protein gene amino acid substitutions and, as markers, ablate naturally-occurring restriction enzyme sites.

rPIV3 designation	Amino Acid Substitution (wt to cp45)	Sequence of wt ^a	Sequence of Mutant	Restriction Enzyme site Ablated
r942	Tyr-942 to His	11468- <u>TTACATGGCCAT</u> (SEQ ID NO: 25)	11468- <u>TCACATGGCGAT</u> (SEQ. ID NO: 26)	Eae I
r992	Leu-992 to Phe	11618- <u>TTTGTGATTGGGC</u> (SEQ ID NO: 27)	11618- <u>TTTGTGATTGGGC</u> (SEQ. ID NO: 28)	Bsr I
r1558	Thr-1558 to Ile	13307-TGG <u>CCCTAAATACTG</u> (SEQ. ID NO: 29)	13307-TGG <u>CCCTAAATA<u>TCG</u></u> (SEQ. ID NO: 30)	Ava II

a. The nucleotide sequence around each of the three mutated regions is shown. The first nucleotide in each provided sequence is numbered according to its position in the complete antigenomic RNA. The codon involved in each amino acid substitution is in bold. Naturally-occurring restriction enzyme sites present in the wt sequence, and which were ablated to mark the mutation, are in italics. The nucleotides that were mutated to produce an aa substitution or remove a restriction enzyme site are underlined.

Mutations introduced in pUCL(N-S) derivatives were verified by dideoxynucleotide sequencing of plasmid DNA. The *Sph* I to *Bam*HI (nt 13733) fragment of pUCL(N-S) containing the cp45 individual L gene mutations was subcloned into the *Sph* I to *Bam*HI sites of pTM(L) to give pTM(L)-942, -992, -942/992, and -1558; the other double and triple mutations were assembled using the *Pin* AI and *Nhe* I sites (Fig. 12). The mutant pTM(L) plasmids were each tested at permissive temperature (32°C) for the ability to direct the expression of the chloramphenicol acetyl transferase marker gene in a minireplicon system comprising a plasmid-encoded minigenome RNA and the N, P and L proteins (Durbin et al., *Virology* 234:74-78 (1997), incorporated herein by reference). The various mutant L plasmids supported marker gene expression to 75-106% the level of wt L, indicating that each engineered cDNA was free of significant spurious mutation (not shown). The *Sph* I to *Nhe* I fragments of each of the mutant pTM(L) plasmids were then subcloned into the *Sph* I to *Nhe* I window of the full-length PIV3 JS antigenomic cDNA p3/7(131)2G to create seven full-length PIV3 cDNA clones representing every possible combination of the three substitutions.

Recovery of recombinant PIV3 (rPIV3) bearing one, two or three cp45 L protein substitutions.

Each full-length antigenomic cDNA bearing one or more cp45 L gene mutations, together with the three support plasmids pTM(N), pTM(P) and pTM(L), was transfected into HEp-2 cells on 6-well plates (Costar, Cambridge, MA) using LipofectACE (Life Technologies) and MVA-T7 as described above. After incubation at 32°C for 4 days, the transfection harvest was passaged onto HEp-2 cells on 6-well plates which were incubated at 32°C for 4 days. Each passage 1 supernatant was harvested and passed onto a T-25 flask of LLC-MK2 cells, which was incubated at 32°C for 5-6 days. The passage 2 supernatant was harvested and the presence of recombinant virus was initially confirmed by immunoperoxidase staining of virus plaques (Murphy et al., *Vaccine* 8:497-502 (1990), incorporated herein by reference) with anti-HN monoclonal antibody (Mab) 77/5, which binds to both biologically derived and recombinant

JS PIV3, and Mab 423/6, which does not bind to cDNA-derived virus because its epitope was ablated to serve as a marker. Virus present in passage 1 was subjected to two or three rounds of plaque purification on LLC-MK2 cells as described previously. Each biologically cloned recombinant virus was amplified twice in LLC-MK2 cells at 32°C to produce virus for further characterization. Virus was concentrated from clarified medium by polyethylene glycol precipitation, and viral RNA (vRNA) was extracted with Trizol Reagent (Life Technologies). Reverse transcription (RT) was performed on vRNA using the Superscript II kit with random hexamer primers (Life Technologies). The Advantage cDNA PCR kit (Clontech, Palo Alto, CA) and sense (5' nt 11190-GCATTATCTAGATGTGTCTTCTGGTCAGAG 3' nt-11219) (SEQ ID NO: 31) and antisense (5' nt 14140-CCTGAATTATAATAATTAACTGCAGGTCCT 3' nt-14111) (SEQ ID NO: 32) primers specific for the PIV3 L gene were used to amplify the region spanning the *Sph* I to *Nhe* I fragment. The PCR fragments were analyzed by digestion with each of the restriction enzymes whose recognition sites had been ablated during insertion of the three cp45 mutations in L (see Table 5).

Efficiency of plaque formation (EOP) at permissive and restrictive temperatures of rPIV3 bearing one, two or three cp45 L protein amino acid substitutions.

The level of temperature sensitivity of plaque formation *in vitro* of control and recombinant viruses was determined at 32°C, 37°C, 38°C, 39°C, 40°C, and 41°C in LLC-MK2 monolayer cultures and plaques were enumerated by hemadsorption with guinea pig red blood cells following removal of the methylcellulose overlay. Alternatively, viral plaques present in the monolayer were identified by immunoperoxidase staining with a mixture of two PIV3-specific anti-HN murine mAbs 101/1 and 454/11 diluted 1:500, (Murphy et al., supra, (1990)).

Hamster studies.

4 to 16 week-old golden Syrian hamsters in groups of six were inoculated intranasally with 0.1ml OptiMEM1 per animal containing 10^{5.5} pfu of rPIV3 JS wt, PIV3 cp45 virus, or

one of the rPIV3 containing one or more cp45 L protein substitution(s). On day 4 post-infection, the hamsters were sacrificed, the lungs and nasal turbinates were harvested, and the virus was quantified as described above. The mean \log_{10} 5 TCID₅₀/g was calculated for each group of six hamsters.

RESULTS

Introduction of the PIV3 cp45 L protein amino acid substitutions into wt JS rPIV3.

As noted above, the three amino acid substitutions present in the L protein of cp45 (Table 5) were introduced individually or in selected combinations into the antigenomic cDNA that encodes its wt parent, PIV3 JS strain. Each introduced mutation was engineered to be marked with a silent mutation that ablated a proximal, naturally-occurring restriction enzyme site to facilitate monitoring the mutation in recovered rPIV3 (Table 5, Fig. 12). The coding change at amino acid 1558 was designed to contain two nucleotide changes in r1558, compared to the one nt substitution in cp45, to reduce the chance of reversion at this site during *in vitro* or 10 15 20 *in vivo* replication.

Seven rPIV3s bearing one, two or all three of the amino acid substitutions from cp45 were recovered in tissue culture by transfection of each antigenomic cDNA together with the pTM(N), pTM(P) and pTM(L) support plasmids and coinfection 25 with the vaccinia virus MVA/T7 pol recombinant (Wyatt et al., *supra*, (1995)). Each rPIV3 possessed the Mab resistance marker that had been deliberately introduced into the HN gene by engineering the antigenomic cDNA. The rPIV3s were biologically cloned by two or three cycles of plaque to plaque 30 passage to ensure that each virus preparation was genetically homogeneous. This precaution was taken because vaccinia virus can mediate recombination between the antigenomic cDNA and the support plasmids.

To confirm that each of the seven rPIV3 contained the engineered mutation(s) in the L gene, RNA was purified from precipitated virions and was copied into cDNA and amplified by RT-PCR. Control reactions showed that the RT step was required for generation of RT-PCR products,

indicating that an RNA template rather than contaminating cDNA was required for the generation of the RT-PCR product. The RT-PCR products were subjected to digestion with the three restriction enzymes whose recognition sequences had been ablated as markers for the inserted coding changes. As expected, the RT-PCR product of JS wt rPIV3 was cleaved the appropriate number of times by each of the three enzymes, whereas r942/992/1558 lacked each of the three sites ablated during creation of the individual cp45 coding changes. Each of the other rPIV3s lacked the appropriate restriction site(s), indicating the presence of the introduced mutations.

Efficiency of plaque formation at 32°C, 37°C, 38°C, 39°C, 40°C, and 41°C of rPIV3 bearing cp45 L mutations.

The seven rPIV3s bearing the various combinations of cp45 L protein amino acid substitutions were assayed for their ability to form plaques on LLC-MK2 monolayers at 32°C, 37°C, 38°C, 39°C, 40°C and 41°C. As shown in Table 6, each rPIV3 bearing a cp45 aa substitution was ts, whereas the JS wt rPIV3 parent was not restricted in plaque formation at any temperature tested. The shut-off temperature of plaque formation of r942, r992 and r1558 was 40°C. r942 manifested a 700-fold reduction of plaque formation at 40°C, indicating that its replication was marginally reduced at this restrictive temperature. However, the plaque size of r942 also was greatly reduced at 40°C, which also indicates that its replication was restricted at this temperature compared to the JS wt. r942 was completely restricted in replication at 41°C (data not shown). r992 and r1558 were greatly reduced (over a 1,000,000-fold reduction) in plaque formation at 40°C. These results indicate that each of the three cp45 L gene mutations individually specifies the ts phenotype, although that of the r942 mutation is somewhat less restrictive. The double mutant virus r942/1558 and the triple mutant r942/992/1558 had a shut-off temperature of 39°C, while that of r942/992 and cp45 was 38°C. The double mutant, r992/1558, was less ts than the r992 single mutant. r992/1558, like r942, was completely restricted in plaque formation at 41°C. The level of temperature sensitivity exhibited by double or

triple results from a rPIVs delicate interplay of the three mutations that cannot be predicted from level of temperature-sensitivity exhibited by the single mutants. Also, since r942/992/1558 was slightly less *ts* than cp45, other mutations outside of the L gene also likely contribute to the *ts* phenotype of cp45, therefore representing additional mutations of interest within the invention.

Table 6: The efficiency of plaque formation (EOP) at 32°C, 37°C, 38°C, 39°C and 40°C of rPIV3 bearing one, two or three cp45 L protein amino acid substitutions.

Virus ^a	Virus titer (\log_{10} pfu/ml)				
	32°C	37°C	38°C	39°C	40°C
r942	6.8	6.8 ^b	6.6 ^b	6.5 ^b	<u>4.3^b</u>
r992	6.9	6.8 ^b	6.7 ^b	6.1 ^b	<0.7
r1558	6.6 ^b	6.6 ^b	6.4 ^b	5.0 ^b	<0.7
r942/992	6.7	6.5 ^b	<u>4.5^b</u>	3.0 ^b	<0.7
r942/1558	5.2	5.0 ^b	4.0 ^b	1.0 ^b	<0.7
r992/1558	6.7	6.7 ^b	6.5 ^b	5.9 ^a	5.1 ^b
r942/992/1558	6.6	6.2 ^b	6.2 ^b	<u>2.7^b</u>	<0.7
cp45 ^b	6.6	4.8	<u>4.5^b</u>	<0.7	<0.7
rPIV3 JS	8.3	8.4	8.5	8.5	8.3

a. The cp45 virus is a biologically derived virus, and each of the other viruses tested is a recombinant.

b. Plaques were of pinpoint size.

c. Underlined numbers represent the shut-off temperature of plaque formation, which is defined as the lowest restrictive temperature at which a 100-fold reduction in titer is observed compared to the titer at 32°C.

Growth in hamsters. Groups of six Golden Syrian hamsters were inoculated intranasally with JS wt rPIV3, biologically-derived cp45, or with rPIV3 containing one or more cp45 L protein amino acid substitutions, and virus replication in the lungs and nasal turbinates was determined. In this experiment [Table 7], each of the rPIV3s bearing a single amino acid substitution was restricted in replication in the upper and lower respiratory tract [Table 7]. However, r942, the least *ts* virus, was only marginally suppressed in

replication in the upper and lower respiratory tract in a second experiment. These data demonstrate that two of the three amino acid substitutions contribute to the att phenotype when present as single-lesioned recombinant viruses. However,
5 the r942 mutation indeed contributes to attenuation (e.g., the r942/992 is more attenuated than r992 alone). Thus, each of the amino acid substitutions in L contribute to the att phenotype either acting alone or in concert with another L amino acid mutation. Each of the double mutants was
10 attenuated indicating that loss of any of the three L gene substitutions following replication in vivo still leaves an attenuated virus. This is a partial explanation for the previously observed high level of stability of the *ts* phenotype of the cp45 following replication in vivo. The triple mutant r942/992/1558 was as restricted as cp45 for replication in the upper and lower respiratory tract indicating that the three amino acid substitutions in the L protein are the major contributors to the att phenotype of
15 cp45.

Table 7: The level of replication in the upper and lower respiratory tract of hamsters of rPIV3 bearing one, two or three cp45 L protein amino acid substitutions, compared to JS wt rPIV3 and cp45^a.

	Virus	Mean virus titer (\log_{10} TCID ₅₀ /g \pm S.E ^b)	
		Nasal turbinates	Lungs
5	rPIV3 wt	7.4 \pm .16	5.1 \pm .49
10	r942	6.6 \pm .17	3.0 \pm .78
	r992	4.4 \pm .16	3.1 \pm .11
	r1558	3.8 \pm .40	4.3 \pm .34
	r942/992	<1.5 \pm 0	<1.5 \pm 0
15	r942/1558	2.9 \pm .23	1.8 \pm .17
	r992/1558	5.7 \pm .16	3.2 \pm .57
	r942/992/1558	3.9 \pm .15	<1.5 \pm 0
	cp45	4.1 \pm .27	1.6 \pm .08

a. Groups of six hamsters each were intranasally administered $10^{5.5}$ pfu of virus per animal in an 0.1ml inoculum, and lungs and nasal turbinates were harvested four days later.

b. Standard Error

c. cp45 is a biologically derived virus and the others are recombinant.

To summarize the above results, substitutions at L protein amino acid positions 992 and 1558 each specified a 1,000,000-fold reduction in plaque formation in cell culture at 40°C, while the substitution at position 942 specified a 700-fold reduction. Thus, each of the three mutations individually contributes to the ts phenotype. The triple recombinant which possesses all three L mutations is slightly less ts than cp45, suggesting that there are mutations outside of the L gene in cp45 that also might contribute to its ts phenotype. Two of the three individual mutations in L each contributed to restricted replication in the upper or lower respiratory tract of hamsters, which accounts for the observed stability of ts and att phenotypes of cp45 during replication *in vivo*. Importantly, the level of temperature sensitivity of recombinant vaccine strains *in vitro* was closely predictive of attenuation *in vivo*. The recombinant virus possessing all three mutations was as restricted in replication as the cp45 mutant in both the upper and lower respiratory tract of

hamsters, indicating that the L gene of the cp45 virus is a major attenuating component of this candidate vaccine strain. While each mutation on its own specifies the *ts* phenotype, when placed together they are not simply additive but instead somehow influence each other. The effect of the three mutations together in the triple mutant seemed to ameliorate rather than enhance the level of temperature-sensitivity observed in the two double mutants which were evaluated.

5 Interestingly, this should provide an unanticipated selective pressure to maintain at least some of the cp45 L mutations, since the loss by reversion of either the 992 or 1558 substitution would increase rather than decrease the level of temperature sensitivity. Considered together, these findings indicate that the high level of the stability of the *ts* and att phenotypes of cp45 virus results from the contribution of multiple *ts* mutations in L to the att phenotype. The identification of these three mutations as the major attenuating mutations of cp45 provides the basis for monitoring virus during all stages of manufacture and

10 following replication in humans.

It is of further interest that the tyrosine to histidine mutation at position 942, arguably the most conservative substitution of the three mutations, was the least temperature sensitive. The L polymerase of PIV3 is a large polypeptide, 2233 aa in length, and is thought to be a multifunctional protein that encodes multiple domains including those required for complex formation with the P protein, RNA binding, RNA polyadenylation, RNA transcription and RNA replication (Collins et al., supra, (1996)). The 15 amino acid substitutions in L at positions 942 and 992 are located near regions that are well-conserved among other members of the Paramyxovirus family (Blumberg et al., Virology 164:487-497 (1982); Galinski et al., Virology 165:499-510 (1988)). The mutation at position 1558 is in a region of the 20 polymerase which appears to share less sequence identity with other L polymerases. Although the mechanism by which the *ts* phenotype is conferred by the triple amino acid substitution in L is not known, it is likely that multiple L protein

domains and activities are affected, or that a common mechanism involving various activities of L is affected.

EXAMPLE X

5 Direct Identification, and Reconstitution into Recombinant Vaccine Viruses, of Mutations in a Biologically Derived, Live-Attenuated HPIV Type 3 Virus (cp45) Which Specify the Temperature-Sensitive, Cold-Adaptation and Attenuation Phenotypes

10 The above Examples demonstrate that each of the three amino acid substitutions in the L polymerase protein of cp45 confer the temperature-sensitive (*ts*) and attenuation (*att*) phenotypes, but not the cold-adaptation (*ca*) phenotype (see also, Skiadopoulos et al., J. Virol. 72(3):1762-8, 1998). cp45 contains twelve additional mutations in other proteins (N, C, M, F and HN) or potential cis-acting sequences (the leader region and the transcription gene start {GS} signal of the N gene), and their contribution to these phenotypes has been heretofore undefined. The present Example further characterizes the genetic basis for the *ts*, *ca*, and *att* phenotypes of cp45 to provide yet additional information regarding basis for the observed high level of stability of these phenotypes following replication of cp45 in humans or non-human primates. In one aspect of this study, a recombinant cp45 (*rcp45*) virus containing all fifteen cp45-specific mutations was constructed, using a reverse genetics system, and was found to be essentially indistinguishable from the biologically-derived virus on the basis of plaque size, level of temperature-sensitivity, cold-adaptation, and level of replication in the upper and lower respiratory tract of hamsters. In addition, recombinant viruses containing: (1) the cp45-specific changes in the C, M, F or HN proteins, (2) the combined leader and N gene mutations, or (3) several combinations of the cp45 mutations were constructed. Analysis of these recombinant viruses showed that multiple cp45 mutations distributed throughout the genome contribute to the *ts*, *ca*, and *att* phenotypes. The mutations in C and F were not *ts* at 40°C but nonetheless

conferred the att phenotype, and they, therefore, are non-ts att mutations. The HN mutation did not confer the ca, ts or att phenotypes. Viruses possessing the 3' leader and N mutations were ts, but exhibited only marginal attenuation in
5 the lower respiratory tract of hamsters. Recombinants possessing several combinations of mutations exhibited a greater level of temperature sensitivity than cp45, but the increased level of temperature-sensitivity was not reflected in an increase in attenuation *in vivo*. These latter findings
10 indicate that the multiple mutations identified in cp45 are interacting to affect replication *in vitro*. The presence of multiple ts and non-ts attenuating mutations in cp45 likely contributes to its high level of attenuation and phenotypic stability. Knowledge of the phenotypes associated with the various mutations of cp45 provided herein allows for accurate monitoring of biologically derived PIV viruses and ready manipulation of recombinant virus to achieve a large
15 assemblage of useful vaccine recombinants within the invention.

20 **Viruses and cells.**

The rPIV3s, PIV3 JS wt and cp45 viruses described in the present Example were grown in simian LLC-MK2 cells (ATCC CCL 7.1) as described above (see also, Durbin et al., Virology 235:323-332, 1997a; Hall et al., Virus Res. 22(3): 173-184,
25 1992; Skiadopoulos et al., J. Virol. 72(3):1762-8, 1998). The modified vaccinia virus Ankara was provided as described above. HEp-2 (ATCC CCL 23) and LLC-MK2 cells were maintained in OptiMEM I (Life Technologies, Gaithersburg, MD) supplemented with 2% FBS and gentamicin sulfate (50ug/mL), or
30 in EMEM supplemented with 10% FBS, gentamicin sulfate (50ug/mL), and 2mM glutamine. L-132 cells (ATCC CCL 5) were grown in Earl's MEM (Life Technologies) supplemented with 10% FBS, 2mM glutamine, 20 mM Hepes, 1 mM non-essential amino acids, and 100 units streptomycin-neomycin/ml.

35 **Construction of point mutations in PIV3.**

Subgenomic fragments of p3/7(131)2G, the antigenomic cDNA clone of PIV3 JS wt used above to recover infectious virus (see also, Durbin et al., Virology 235:323-332, 1997a;

Skiadopoulos et al., *J. Virol.* 72(3):1762-8, 1998), were cloned into pUC19 vectors modified to accept these fragments, using standard molecular cloning techniques. Point mutations corresponding to mutations identified in cp45, as well as mutations creating or ablating silent restriction enzyme recognition sequences (Table 8) were introduced using the Transformer Mutagenesis Kit (Clontech, Palo Alto, CA) as described previously.

Table 8: PIV3 cp45 mutations introduced into rPIV3

	seq id no	region affected	nt position n ^a	sequence changes ^b	restriction marker ^c	amino acid substitution ^d
1	46 47	3' leader	20	TTGTCTGGGAAT <u>TTGC</u> CTGGGAAT	none	non-coding
2	46 48	3' leader	20	TTGTCTGGGAAT TTG <u>T</u> CTGGGAAT	none	non-coding
3	46 49	3' leader	20	TTGTCTGGGAAT TTGTCTGG <u>A</u> AT	none	non-coding
4 ^e	50 51	3' leader	40	AACTTTAATT <u>A</u> AACT <u>T</u> AAATT <u>A</u>	-Dra I	non-coding
5 ^e	52 53	N gene start	60	TTAAAGACATTG TT <u>A</u> AGACATTG	none	non-coding
6 ^e	54 55	N	390	GCAGATGTCAAG GCAGAT <u>G</u> CAAG	none	Val-96 to Ala
7 ^e	56 57	N	1271	CGAAC <u>T</u> AAAGA CGAAC <u>G</u> CTAAAGA	none	Ser-389 to Ala
8	58 59	C	2076	GAA <u>A</u> ATTGATC GAA <u>A</u> ACATTGATC	-Ssp I	Ile-96 to Thr
9	60 61	M	4341	TCTCTACCCAAC <u>TCG</u> TTAACCAAC	+Hpa I	Pro-199 to Thr
10 ^f	62 63	F	6323	AGTACAATAGGT AGTACT <u>G</u> GGGT	+Sca I	Ile-420 to Val
11	64 65	F	6419	<u>GC</u> ACTTGATCCA ACACT <u>G</u> GATCCA	+Bam HI	Ala-450 to Thr
12	66 67	HN	7944	CCATCAT <u>T</u> GGTGTGACAA CCATCAT <u>T</u> GGCTGACAA	+Bst XI	Val-384 to Ala
13	68 69	L	11468	TTACATGGCCA <u>TC</u> ACATGGCGA	-Eae I	Tyr-942 to His
14	70 71	L	11618	TTGGGACTGGGC <u>TTT</u> TGATTGGGC	-Bsr I	Leu-992 to Phe
15 ^f	72 73	L	13308	<u>GG</u> TCTTAATACT GGCCTTAATATC	-Ava II	Thr-1558 to Ile

a. Position of the first nucleotide in the PIV3 sequence shown. b. Wild type sequence is shown above the mutant sequence. Nucleotide changes are underlined. Codon substitutions are in bold font. c. Each relevant restriction endonuclease recognition sequence is in italics; (+) indicates addition of new restriction endonuclease recognition sequence; (-) indicates ablation of a naturally occurring restriction endonuclease recognition sequence. d. Mutations are indicated as the three letter amino acid assignment of wt, followed by the amino acid position, followed by the cp45 assignment. e. These mutations were identified by Joanne Tatem (unpublished observations), the others were from Stokes et al., *Virus Res.* 30(1):43-52, 1993. f. Two nucleotides were changed in the indicated codon in order to reduce the chance of reversion to wild type sequence.

After mutagenesis, restriction endonuclease fragments were sequenced completely and cloned back into the full-length clone, p3/7(131)2G. The 3' leader and N mutations were amplified by reverse transcription (RT)-PCR directly from PIV3 cp45 virion RNA and were cloned into pLeft+2G (see above), or a modified pUC19 vector for further manipulation. Combinations of mutations were constructed using standard molecular cloning techniques. The full-length plasmid clone containing the cp45 mutations, designated pFLCcp45, was completely sequenced to determine if extraneous mutations had been introduced during the cloning process, but none were found.

Recovery of recombinant PIV3 (rPIV3).

Each full-length antigenomic cDNA bearing cp45 mutations, together with the three support plasmids pTM(N), pTM(P no C) and pTM(L), was transfected into HEp-2 cells on 6-well plates (Costar, Cambridge, MA) using LipofectACE (Life Technologies, Gaithersburg, MD) and MVA-T7 as described above. After incubation at 32°C for 4 days, the transfection harvest was passaged onto LLC-MK2 cells in T-25 flasks which were incubated at 32°C for four to eight days. This harvested virus was called passage 1 and was subjected to three rounds of plaque purification on LLC-MK2 cells as described above. Each biologically-cloned recombinant virus was amplified twice in LLC-MK2 cells at 32°C to produce virus for further characterization. Virus was concentrated from clarified medium by polyethylene glycol precipitation (see, Mbiguino and Menezes, J. Virol Methods 31:2-3, 1991, incorporated herein by reference in its entirety), and viral RNA (vRNA) was extracted with Trizol Reagent (Life Technologies). Reverse transcription was performed on vRNA using the Superscript II Preamplification System (Life Technologies) with random hexamer primers. The Advantage cDNA PCR kit (Clontech) and sense and antisense primers specific for various portions of the PIV3 genome were used to amplify fragments for restriction endonuclease analysis. The PCR fragments were analyzed by digestion with each of the restriction enzymes whose

recognition sites had been created or ablated during construction of the mutations (Table 8).

5 **Efficiency of plaque formation (EOP) of rPIV3 bearing cp45 mutations at permissive and restrictive temperatures.**

The level of temperature-sensitivity of plaque formation *in vitro* of control and recombinant viruses was determined at 32°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, and 41°C in LLC-MK2 monolayer cultures as described above (see also, 10 Hall et al., Virus Res. 22(3):173-184, 1992, incorporated herein by reference in its entirety). Plaques were enumerated by hemadsorption with guinea pig red blood cells following removal of the methylcellulose overlay, or alternatively, the viral plaques present in the monolayer were identified by 15 immunoperoxidase staining with a mixture of two PIV3-specific anti-HN murine mAbs 101/1 and 454/11 diluted 1:250 (see, Murphy et al., Vaccine 8(5):497-502, 1990; van Wyke Coelingh et al., Virology 143(2):569-582, 1985).

20 **Evaluation of rPIV3 mutant viruses for cold-adaptation phenotype.**

Growth of mutant and wt rPIV3 viruses was determined at 32°C and 20°C on confluent L-132 cell monolayers prepared in 24-well tissue culture plates. Duplicate wells of each of two plates were inoculated with 0.2 ml of each mutant or wt rPIV3 25 virus at a multiplicity of infection of 0.01. After one hour adsorption at room temperature, the inoculum was aspirated and the monolayers were washed with 1 ml of PBS per well. The inoculated cultures were overlaid with 0.5 ml of Earl's MEM supplemented with 10% FBS, 2mM glutamine, 20 mM Hepes, 1 mM 30 non-essential amino acids, and 100 units streptomycin-neomycin/ml. One plate was sealed in a waterproof pouch (Kapak) and then submerged in a 20°C bath for 13 days. The duplicate plate was placed at 32°C in a CO₂ incubator for 3 days. At the end of the incubation period, 35 virus was harvested by freeze/thawing. The titer of virus recovered from each well was determined by plaque assay in LLC-MK2 cells at 32°C using hemadsorption with guinea pig red

blood cells to visualize plaques. Two wt and two cp45 reference stocks were used as controls.

Hamster studies.

5 week-old Golden Syrian hamsters in groups of five
5 were inoculated intranasally with 0.1 ml OptiMEM I per animal
containing $10^{6.0}$ pfu of JS wt rPIV3, PIV3 cp45 virus, or one
of the mutant rPIV3s. On day 4 post-infection, the hamsters
were sacrificed, the lungs and nasal turbinates were
harvested, and the virus was quantified as described above.
10 The mean \log_{10} TCID₅₀/gram at 32°C was calculated for each group
of hamsters.

RESULTS

Introduction of the PIV3 cp45 mutations into JS wt rPIV3.

The fifteen mutations in the 3' leader, the N GS signal, and the N, C, M, F, HN and L proteins of cp45 (Table 8) were introduced into the complete PIV3 antigenomic cDNA by site directed mutagenesis or by direct PCR amplification of a segment of cp45 cDNA bearing the desired mutations. The following antigenomic cDNAs were made (see Fig. 13): (i) rcp45 3'N, containing the four point mutations of the leader region, the point mutation in the N GS signal, and the two amino acid changes in the N protein; (ii) rcp45 C, containing the single amino acid change in the C protein; (iii) rcp45 M, containing the single amino acid change in M, (iv) rcp45 F, containing the two amino acid changes in F; (v) rcp45 HN, containing the single amino acid change in HN; (vi) rcp45 L, containing the three amino acid changes in L, as described above; (vii) rcp45 3'NL, containing the mutations from i. and vi. above; (viii) rcp45 3'NCMFHN, containing all of the mutations except for the three in L; and (ix) rcp45, containing all fifteen cp45 mutations listed in Table 8. In most cases, each cp45 change was engineered to be accompanied by one or more nearby silent changes which introduced or removed a restriction enzyme recognition site (Table 8). These served as markers to confirm the presence of the mutation in the cDNA and in recovered virus. Also, two of the amino acid coding changes (mutation numbers 10 and 15 in Table 8) were made using two

nucleotide changes rather than the single change found in cp45, reducing the possibility of same-site reversion to wt. The cp45 cDNA, which contains all fifteen of the cp45 changes in Table 8, was assembled from the same mutagenized cDNA subclones as were used to introduce cp45 changes into the other antigenomic cDNAs; it was sequenced in its entirety, and was confirmed to contain only the intended mutations. This indicated that all of the regions which had been subjected to mutagenesis or PCR and had been manipulated by cloning possessed the desired sequences and lacked other unwanted mutations. Each full-length plasmid bearing one or more of the cp45 mutations was transfected into HEp-2 cells along with support plasmids and MVA-T7 to produce recombinant PIV3 as described above. Analysis of RT-PCR fragments encompassing the mutations indicated in Table 8 were amplified from virion RNA of the various recombinant viruses indicated in Figure 13 confirmed the presence of the introduced mutations, and other unintended mutations were not found.

Plaque morphology.

Several of the recombinant viruses exhibited distinctive plaque morphologies when tested on LLC-MK2 cells. JS wt rPIV3 plaques averaged 1mm in size, and were indistinguishable in size from the biologically derived JS wt PIV3. Plaques of the cp45 and rcp45 viruses were larger than wt, averaging two- to three-fold larger in diameter than wt, and were indistinguishable from each other. This demonstrated the comparability of the biologically-derived and recombinant cp45 virus for this phenotype.

Efficiency of plaque formation of rPIV3s bearing the cp45 mutations in LLC-MK2 cells at permissive and restrictive temperatures.

The rPIV3s were assayed for their ability to form plaques at permissive and restrictive temperatures ranging from 32°C to 41°C (Table 9). Analysis of the ts phenotypes of viruses bearing individual components of cp45 revealed that the rcp45 3'N and rcp45 M viruses had a shutoff temperature of 40°C, and the rcp45 C mutant had a shutoff of 41°C. The shutoff temperature of rcp45 F and rcp45 HN mutants was

greater than 41°C. Consistent with the above results, the
rcp45 L virus had a shutoff temperature of 39°C. A virus is
considered to have a ts phenotype, for example, if its
reduction of replication at 40°C (ie. titer at 32°C minus titer
5 at 40°C) is approximately \geq 100-fold that of wt virus at 40°C.
Applying this definition, the present results indicated that
at least two regions of cp45 (3'N, and L) contribute to the ts
phenotype.

66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

Table 9: The efficiency of plaque formation (EOP) of recombinant and biologically-derived viruses at permissive and restrictive temperatures.

Virus	Mean Virus Titer ^a (\log_{10} Pfu/ml)						<i>ts</i> phenotype
	32°C	35°C	36°C	37°C	38°C	39°C	
rcp45 3'N	7.1	---	---	7.0	6.4	5.4	4.2
rcp45 C	6.9	---	---	7.0	6.7	6.6	5.9
rcp45 M	7.7	---	---	7.4	7.0	6.5	5.3
rcp45 F	7.5	---	---	7.2	6.0	6.6	5.9
rcp45 HN	6.4	---	---	6.5	6.2	6.4	4.7
rcp45 L	7.3	---	---	7.2	6.7	4.0	<0.7
rcp45 3'NL	7.3	5.7	<0.7	<0.7	<0.7	<0.7	<0.7
rcp45 3'NCMFHN	7.2	5.6	<0.7	2.0	2.4	<0.7	<0.7
rcp45	8.5	7.5	7.1	6.4	6.0	2.0	<0.7
cp45^b	8.3	8.0	7.4	7.0	6.2	2.3	<0.7
rPIV3 wt	7.3	7.3	7.0	7.4	7.6	7.7	6.0

a. Plaques were enumerated by immunoperoxidase staining after incubation for 6 days at the indicated temperature. Values are the mean of two experiments, values in bold are from a single experiment. Underlined values represent the lowest non-permissive temperature at which a 100-fold reduction of plaquing efficiency occurs compared to titer at 32°C, and this is defined as the shutoff temperature of plaque formation. b. cp45 is biologically-derived and the other viruses are recombinants. c. A virus is defined as bearing the *ts* phenotype if its reduction of replication at 40°C (ie. titer at 32°C minus titer at 40°C) is \geq 100-fold that of wt virus at 40°C.

As shown in Table 9, rcp45, containing all of the cp45 mutations, had a shut-off temperature of 38°C, which was identical to that of the biologically-derived cp45. These results show that the ts phenotype of cp45 was successfully reproduced in rcp45. In addition, these results validate the sequence analysis of cp45 and the subsequent reconstruction of mutations into recombinant virus.

The rcp45 3'NCMFHN virus, which is identical to rcp45 except that it lacks the three L mutations, exhibited a shutoff temperature of 36°C. Since the L mutations are known to confer temperature-sensitivity individually and in combination, it is somewhat paradoxical that rcp45 3'NCMFHN was more, rather than less, ts than cp45. This implies that there is an interaction of mutations within cp45 whereby mutations compensate for each other to give a level of temperature-sensitivity which is less than the sum of the individual components.

Virus rcp45 3'NL was constructed to investigate whether the L mutations interact with the leader and/or N mutations, since all of these elements are believed to interact during RNA synthesis. This virus had a shutoff temperature 36°C, compared to 40°C and 39°C for rcp45 3'N and rcp45 L, respectively. These results suggest that there is an interaction between the 3'N and L mutations that results in augmentation of temperature-sensitivity. These results also provide another example in which a subset of cp45 mutations specifies a level of temperature sensitivity greater than that observed for rcp45 which contains the entire set of mutations.

ca phenotype of rPIV3s bearing cp45 mutations.

The rPIV3s were analyzed to determine which genetic elements of cp45 specified the ca phenotype (Table 10). It was demonstrated above that rcp45 L is ts and att, but not ca. This indicates that the genetic elements specifying the greater part of the ca phenotype are located outside L, and this was confirmed in the present study. Each of the rPIVs possessing the 3' leader and N mutations were ca except rcp45

3'NCMFHN, which exhibited an intermediate phenotype. This shows that the ca phenotype is specified mostly within the 3'N region. The finding that the level of ca of viruses containing the 3'N segment is less than that of cp45 or rcp45 indicates that other regions of cp45 contribute to the ca phenotype, even though this is not apparent from analysis of the other regions individually. The rcp45 3'NL virus is more ca than the rcp45 3'N virus, suggesting that the L mutations may make a modest contribution. The biologically-derived cp45 and rcp45 viruses exhibit comparable levels of ca, indicating that this phenotype, like the plaque size and ts phenotypes, was successfully reproduced in the recombinant cp45 virus provided herein. Therefore, the ca phenotype, like the ts phenotype, is a composite phenotype reflecting individual contributions to the overall phenotype as well as contributions from interacting genetic elements.

Table 10 : Growth of wt and mutant PIV3s at 20°C compared to 32°C^a (cold adaptation phenotype).

Virus	20°C	32°C	ca	Phenotype
cp45 ^b	6.72	8.49	+	
rcp45	6.57	8.35	+	
rcp45 3'NL	5.02	7.23	+	
rcp45 3'N	4.39	8.53	+	
rcp45 3'NCMFHN	3.53	8.08	+/-	
rcp45 L	3.18	7.98	-	
rcp45 C	2.52	8.32	-	
rcp45 F	2.47	8.10	-	
rcp45 HN	2.11	8.01	-	
rcp45 M	1.76	8.21	-	
PIV3 WT	2.57	8.43	-	

^a. Virus titer is expressed in log₁₀ PFU/mL. ^b. cp45 is biologically-derived and the other viruses are recombinant. The ca phenotype is defined as a greater than 10-fold increase in growth at 20°C relative to wt.

Growth of the rcp45 mutant viruses in hamsters.

Groups of five Golden Syrian hamsters were inoculated intranasally with 10⁶ TCID₅₀ of recombinant or

biologically-derived virus, and the level of virus replication in the lungs and nasal turbinates was determined four days later (Table 11). The fourth day post-inoculation has been shown previously to be the peak of virus replication in hamsters for both the wt and *cp45* viruses (see, Crookshanks and Belshe, J. Med Virol 13(3):243-9, 1984, incorporated herein by reference in its entirety). The *rcp45* virus was reduced approximately 40-fold in replication in the nasal turbinates and 1000-fold in the lungs, and thus was as attenuated as the biologically-derived *cp45* virus. These results indicate that the attenuation phenotype of *cp45* was successfully reproduced in its recombinant version. Since each phenotype of *cp45* was fully reproduced in *rcp45*, the additional five mutations in *cp45* that were not included in this Example likely made little contribution to the properties of *cp45*.

65 22 22 22 22 22 22
25

Table 11: Level of replication in the upper and lower respiratory tract of hamsters^a of wt and mutant PIV3s^b.

Virus	Mean virus titer (\log_{10} TCID ₅₀ /g \pm S.E ^c) in:	
	Nasal turbinates	Lungs
JS wt rPIV3	6.9 \pm 0.2	5.4 \pm 0.5
rcp45 3'N	6.5 \pm 0.2	3.9 \pm 1.1
rcp45 C	4.8 \pm 0.3	3.1 \pm 0.7
rcp45 M ^d	6.8 \pm 0.2	6.7 \pm 0.3
rcp45 F	4.6 \pm 0.2	3.4 \pm 0.6
rcp45 HN	6.3 \pm 0.2	5.3 \pm 1.0
rcp45L	4.2 \pm 0.1	2.1 \pm 0.3
rcp45 3'NL	4.7 \pm 0.2	2.1 \pm 0.3
rcp45 3'NCMFHN	5.8 \pm 0.3	2.9 \pm 0.9
rcp45	5.3 \pm 0.1	2.4 \pm 0.2
cp45	4.9 \pm 0.4	1.9 \pm 0.2

a. Groups of five hamsters each were intranasally administered $10^{6.0}$ TCID₅₀ of virus per animal in an 0.1ml inoculum, and lungs and nasal turbinates were harvested four days later. b. cp45 is a biologically-derived virus, the other viruses are recombinant. c. TCID₅₀, 50% tissue infectious dose \pm Standard Error. d. The virus pool used in this study was found to contain a mixed plaque phenotype. The attenuation phenotype of this mutant will be reassessed using additional virus preparations.

As demonstrated above, the mutations in the L gene of cp45 specify the majority of the attenuation phenotype of this virus. In the present Example, the contribution of the cp45 mutations outside of L as a group was examined. The rcp45 3'NCMFHN mutant was only slightly reduced in replication in the nasal turbinates, but was more than 100-fold reduced in replication in the lungs, which shows that additional attenuating mutations were present outside of the L protein. Importantly, if each of the three mutations in the L gene of rcp45 reverted to wild type sequence, the resulting virus, rcp45 3'NCMFHN, would still retain the attenuation phenotype. The rcp45 M and rcp45 HN mutant viruses were not defective for replication in the respiratory tract of hamsters, and the rcp45 3'N virus showed only a marginal decrease in replication

in the lower respiratory tract. This suggests that the mutations present in the 3' leader, in the N gene start site and the N, M and HN proteins are not attenuating in and of themselves. However, these mutations could contribute to the overall attenuation of cp45 in the context of the other cp45 mutations. Also, individual mutations within the 3'N region may have effects which are not apparent when the set of mutations is analyzed together, which can be readily determined according to the present disclosure.

Replication of the rcp45 C and rcp45 F mutant viruses was approximately 100-fold reduced in both the nasal turbinates and the lungs, demonstrating that the mutations present in the C and F proteins of cp45 confer the attenuation phenotype in hamsters, although the level of attenuation is not as great as that conferred by the cp45 L mutations. As described above, the rcp45 F and rcp45 C mutant viruses did not possess the ts phenotype, and therefore, the mutations that occur in the C and F proteins are considered to be non-ts attenuating mutations.

EXAMPLE XI

Recovery of Recombinant, Chimeric PIV3 in which the Hemagglutinin and Fusion Glycoproteins have been Substituted with

Corresponding Glycoproteins From PIV1

Within the present example, a chimeric rPIV virus is generated and selected which incorporates one or more heterologous genes or large polynucleotide sequences from one PIV into a different rPIV background. Within this aspect of the invention, individual genes or gene segments of one PIV are substituted by counterpart sequence(s) from a heterologous PIV or other source. In one embodiment described in the present Example, tools and methods are provided for substituting, e.g., the HN and/or F protective antigens of HPIV1 or HPIV2 into a recombinant HPIV3 to yield a chimeric recombinant suitable for developing live-attenuated vaccines.

Viruses and cells.

The PIV1 strain used in this study, PIV1/Washington/20993/1964 (PIV1/Wash64), was isolated in Washington DC in 1964 and was confirmed to be a virulent wild type virus in clinical studies in adult human volunteers (Murphy et al. Infect. Immun. 12:62-8 (1975), incorporated herein by reference). It was propagated in LLC-MK2 cells (ATCC CCL 7.1) in Opti-MEM I (Life Technologies) with 50 µg/ml gentamicin sulfate, 2 mM glutamine and 0.75 µg/ml trypsin (Catalog No. 3741, Worthington Biochemical Corp., Freehold, NJ). The Greer strain of human PIV2 (Catalog No. V-322-001-020, NIAID Repository, Rockville, MD) used in the hemagglutination-inhibition assay (HAI) was propagated in the same way. The JS strain of human PIV3 virus and its recombinant derivative from cDNA (rPIV3/JS) with wild type phenotype were propagated as described above. The modified vaccinia Ankara (MVA) recombinant that expresses the bacteriophage T7 RNA polymerase is described in Wyatt et al., Virology 210:202-205 (1995) (incorporated herein by reference).

HEp-2 cells were obtained from ATCC (ATCC CCL 23) and maintained in Opti-MEM I (Life Technologies) with 2% fetal bovine serum (FBS), 50 µg/ml gentamicin sulfate and 2 mM glutamine.

Construction of a cDNA encoding a complete chimeric PIV3-PIV1 antigenome.

A cDNA encoding a full-length PIV3 antigenomic RNA in which the PIV3 HN and F ORFs were replaced by their PIV1 counterparts was constructed as shown in Figure 14. cDNA clones of the HN and F genes of PIV1/Wash64 were prepared from RNA extracted from LLC-MK2 cells which had been infected with PIV1/Wash64 wild type virus. cDNA was generated using the SuperScript Preamplification System using random hexamer primers (Life Technologies). PIV1 F and HN cDNAs were amplified with Vent DNA polymerase (New England Biolabs, Beverly, MA) using gene specific primer pairs based on consensus sequences present in GenBank. All primers described below are annotated so that PIV specific sequences are

underlined, restriction sites are in italics, nt altered from wild type sequences are in lowercase, and start and stop codons are in bold. The positive sense PIV1 F primer, hybridizing to nt 69-97 upstream of the start codon, was
5' -GGGAAAGAAtCCAGAGACAAGAACGG-3' (SEQ ID NO: 33). The negative- sense PIV1 F primer, hybridizing to nt 36-61 downstream of the F stop codon, was
5' -GGTGAAGTTGTGGATccATTGATTG-3' (SEQ ID NO: 34). It carries a *BamH* I site. The positive-sense primer for PIV1 HN was 5' -CAACCTGTAAGGtAcCAGCATCCG-3' (SEQ ID NO: 35). It hybridizes to nt 13-36 upstream the HN start codon and carries a *Kpn* I site.
10 The negative-sense PIV1 HN primer was
5' -GATATGGTGTaGGcCTTGATCTGTTC-3' (SEQ ID NO: 36). It hybridizes to the last two nt of the stop codon and 25 nt further downstream and carries a *Stu* I site. The PIV1 F cDNA was cloned as a *BamH* I and blunt-end fragment into *BamH* I-*EcoR* V digested pLITMUS28 (New England Biolabs), while the PIV1 HN cDNA was cloned as a *Kpn* I-*Stu* I fragment into the same vector. The nt sequences of the resulting plasmids, designated as pLit.1HNwt and pLit.1Fwt (GenBank Accession number: AF016280, AF016279), were determined using the Circumvent Sequencing Kit (New England Biolabs). These two clones were modified (Fig. 14) using mutagenic PCR primers to delete their non-coding regions and to introduce new
25 restriction sites flanking their start and stop codons for the purpose of constructing the PIV3-1 chimeric HN and F genes. The sequences of positive-sense and negative-sense PIV1 F mutagenic primers were 5'-CgccATGgAAAAATCAGAGATCCTCTTCT-3' (SEQ ID NO: 37) and 5' -CtggatccAATTGGAGTTGTTACCCATGTA-3' (SEQ ID NO: 38), respectively, the introduced restriction sites are *Nco* I and *BamH* I. The sequences of positive-sense and negative sense PIV1 HN mutagenic primers were 5' -AACcATGGCTGAAAAAGGGAAA-3' (SEQ ID NO: 39) and 5' -GGTGAaGCTtAAGATGTGATTTACATATTTA-3' (SEQ ID NO: 40),
30 respectively, the introduced restriction sites are *Nco* I and *Hind* III.

The second step involved the modification of the PIV3 HN and F genes to serve as acceptors of the PIV1 coding

regions generated above. The PIV3 F and HN genes were subcloned in several steps from the full-length clone of PIV3/JS, p3/7(131)2G to generate pLit.PIV3.HN.4 and pLit.PIV3.F.3a (Fig. 14). The PIV3 F or HN coding regions were deleted by PCR mutagenesis and replaced by restriction sites appropriate to accept the PIV1 HN and F cDNA described above. The sequences of the PIV3 F positive sense and negative sense mutagenic primers were

5 5'-AAATAggatccCTACAGATCATTAGATATTAAAAT-3' (SEQ ID NO: 41) and
10 5'-CgcCATgGTGTTCAGTGCTTGTG-3' (SEQ ID NO: 42), respectively, the introduced restriction sites were *BamH* I and *Nco* I. The sequences of PIV3 HN positive sense and negative sense
15 mutagenic primers were 5'-CCACAAgCtTAATTAACCATAATGCATCA-3' (SEQ ID NO: 43) and 5'-TTCCATggATTGGATTGTCTATTGGGT-3' (SEQ ID NO: 44), respectively, the introduced restriction sites were *Hind* III and *Nco* I. The stop codon for the chimeric HN would be regenerated upon ligation with mutagenized PIV1 HN cassette. The PIV1 HN or F cDNAs described above were imported in as a *Nco* I-*Hind* III fragment for HN or as a *Nco* I-*BamH* I fragment for F, which generated pLit.PIV3-1.HNhC and pLit.PIV3-1.FhC. These two cDNAs were then joined into pSE.PIV3-1.hC, which was subsequently sequenced in its
20 entirety. Then the *BspE* I-*Sph* I fragment was inserted into p3/7(131)2G to generate pFLC.2G+.hC (Fig. 14). The nucleotide sequence of this *BspE* I-*Sph* I fragment, containing the chimeric F and HN genes, is in GenBank (Accession No.
25 AF016281). Importantly, the cDNA engineering was designed so that the final rPIV3-1 antigenome conformed to the rule of six (Durbin et al. *Virology* 234:74-78 (1997), incorporated herein by reference). The plasmid pFLC.2G+.hC encoding the rPIV3-1 chimera was deposited on 9/17/97 under the terms of the Budapest Treaty with the American Type Culture Collection
30 (ATCC) of 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.

35 **Transfection.** HEp-2 cell monolayers were grown to confluence in six-well plates (Costar Corp, Cambridge, MA) and transfections were performed as described above. Cell monolayers were infected with MVA-T7 at a multiplicity of

infection (MOI) of 3 in 0.8 ml of serum-free Opti-MEM I containing 50 µg/ml gentamicin, and 2 mM glutamine and then transfected with the three support plasmids, 0.4 µg pTM(N), 0.4 µg pTM(P), 0.05 µg pTM(L), and 5 µg the PIV3-1 antigenome cDNA. The plasmids were mixed in 0.2 ml of Opti-MEM I containing 12 µl LipofectACE (Life Technologies) and added to each well. The PIV3/JS cDNA plasmid, p3/7(131)2G, which encodes wild type PIV3/JS antigenomic RNA, was transfected in parallel as a positive control. After incubation at 32°C for 12 hours, the transfection medium was replaced with 1.5 ml of fresh Opti-MEM I supplemented with 50 µg/ml gentamicin, and cultures were incubated at 32°C for two additional days. Trypsin was added to a final concentration of 0.75 µg/ml on day 3 post transfection. Cell culture supernatants were harvested on day 4 and passaged (referred to as passage 1) onto fresh LLC-MK2 cell monolayers. After overnight adsorption, the medium was replaced with fresh Opti-MEM I with 0.75 µg/ml trypsin. Passage 1 cultures were incubated at 32°C for 4 days, and the amplified virus was harvested and further passaged on LLC-MK2 cells (referred to as passage 2) for another 4 days at 32°C in the presence of 0.75 µg/ml trypsin. The recovered virus was characterized by reaction with rabbit PIV1 antiserum, guinea pig PIV2 antiserum, and two PIV3 HN monoclonal antibodies in a hemagglutination-inhibition (HAI) assay as described previously (van Wyke Coelingh et al., supra, (1985)) with the exception that chicken red blood cells (RBCS) were used for viruses possessing the PIV1 HN glycoproteins, whereas guinea pig RBCs were used for PIV3. The HAI was performed to determine if rPIV3 possessed the monoclonal antibody resistant mutation (MARM) that marked virus recovered from cDNA or if rPIV3-1 possesses the PIV1 HN.

Nucleotide sequence analysis. The chimeric cDNA construct pSE.PIV3-1.hc was sequenced with the Circumvent Sequencing kit (New England Biolabs) before the final assembly into full-length clone pFLC.2G+.hc. For analysis of RNA, the appropriate PIVs were amplified in T75 flasks of LLC-MK2 cells. Virus was harvested on day 5 post-infection and concentrated by polyethylene glycol precipitation (Mbiguino et

al., J. Virol. Methods 31:161-70 (1991)). Viral RNA was extracted from the viral pellets and used in reverse transcription (RT) with the Superscript Preamplification System (Life Technologies). RT-PCR was performed using the Advantage cDNA synthesis kit (Clontech) and PIV1 or PIV3 specific primer pairs. RT-PCR products were gel purified by electrophoresis onto, and elusion from, strips of NA45 DEAE nitrocellulose membrane (Schleicher & Schnuell, Keene, NH), and were sequenced.

Replication of PIVs in LLC-MK2 cells. Plaque enumeration on LLC-MK2 monolayers was performed as previously described except that trypsin was added in the case of the PIV1 and rPIV3-1 viruses (Hall et al. Virus Res. 22:173-84 (1992)). Briefly, serially-diluted virus was inoculated onto LLC-MK2 monolayers in six-well plates; virus was adsorbed for 1 hour at 32°C; the cultures were overlaid with L-15 medium (Life Technologies) containing 0.8% agarose with or without added trypsin and incubated at 32°C for 6 days; and plaques were identified by hemadsorption (HAD) with guinea pig red blood cells following the removal of the agarose overlay.

Growth of the PIV viruses in tissue culture was evaluated by infecting confluent LLC-MK2 monolayers on twelve-well plates with virus at a MOI of 0.01. After adsorption at 32°C for 1 hour, the inoculum was replaced with 1.5 ml/well Opti-MEM I supplemented with gentamicin (50 µg/ml) and trypsin (0.75 µg/ml), and further incubated at 32°C for 6 days. At 24 hour intervals, 0.3 ml medium was removed from each well and 0.3 ml fresh medium with trypsin was added back. The titer of virus was determined at 32°C by hemadsorption assay on LLC-MK2 cell monolayers using fluid overlay as previously described (Hall et al. Virus Res. 22:173-84 (1992)), and the titers were expressed as log₁₀TCID₅₀/ml.

Replication of PIVs in the respiratory tract of hamsters. Golden Syrian hamsters in groups of 12 were each inoculated intranasally with 0.1 ml of L-15 medium containing 10⁵ pfu of rPIV3/JS, rPIV3-1, or PIV1/Wash64. On days 4 and 5 post-inoculation, six hamsters from each group were sacrificed, and their lungs and nasal turbinates harvested,

and homogenized, and virus present in the samples was titered on LLC-MK2 cell monolayers at 32°C. The titers were expressed as mean \log_{10} TCID₅₀/g for each group of six hamsters.

5

RESULTS

Construction of a cDNA clone encoding a full-length, chimeric PIV3-1 antigenomic RNA yielded recovery of the Chimeric Virus rPIV3-1.

As noted above, the final construct of the PIV3 and PIV1 chimeric cDNA, in which the ORFs of the JS wild type PIV3 HN and F glycoprotein genes were replaced by those of PIV1/Wash64 coding sequences (Fig. 14) encodes a PIV3-1 chimeric antigenomic RNA of 15,516 nt, which conforms to the rule of six (Durbin et al., *Virology* 234:74-78 (1997)). The pFLC.2G+.hc cDNA encoding the chimeric PIV3-1 antigenome was transfected onto HEp-2 cells together with the N, P and L support plasmids. The p3/7(131)2G cDNA encoding the JS wt PIV3 antigenome was transfected in parallel to generate a rPIV3 control parental virus. Virus was recovered from each transfection following the second amplification on LLC-MK2 cells, and studies were initiated to confirm that each recombinant virus was derived from cDNA.

Recombinant viruses rPIV3-1 and rPIV3 were first characterized for the presence of the PIV1 or PIV3 HN glycoprotein by HAI assay with serotype-specific anti-HN monoclonal or polyclonal antibodies. As shown in Table 12, rPIV3 reacted with only one of the two PIV3 mAbs as expected, whereas its biologically derived parent PIV3/JS reacted with both. This confirmed that rPIV3 contained the introduced MARM mutation that marks this virus as being derived from cDNA. The rPIV3-1 virus reacted with antibodies specific to the PIV1 HN glycoprotein, but not to ones specific to HN of PIV3 or PIV2, showing that the virus contained the PIV1 HN gene as expected.

Table 12. rPIV3-1 possesses the HN glycoprotein of PIV1

Virus	PIV1 ^b antiserum	PIV2 ^b antiserum	α -PIV3 mAb 423/6 ^c	α -PIV3 mAb 77/5 ^c
PIV1/Wash64	256	32 ^d	\leq 50	\leq 50
rPIV3-1	64	\leq 2	\leq 50	\leq 50
rPIV3/JS	4	\leq 2	\leq 50	3,200
PIV3/JS	8	\leq 2	12,800	6,400
PIV2/Greer	8	512	\leq 50	\leq 50

^aChick red blood cells (RBC) were used in HAI assay for PIV1, PIV2, and rPIV3-1 and guinea pig RBCs were used for PIV3/JS and rPIV3/JS.

^bPIV1 rabbit antiserum was purchased from Denka Seiken Co. Ltd., Japan (Catalog No. 410-701), and PIV2 guinea pig antiserum was obtained from NIAID repository, Rockville, MD (Catalog No. V-322-50-558).

^cBiologically derived PIV3/JS contains epitopes recognized by both mAb 423/6 and 77/5, whereas rPIV3/JS was engineered to lack reactivity with mAb 423/6.

^dThe PIV2 antiserum had some reactivity with PIV1 virus, and therefore is not completely type specific.

It was next confirmed that the rPIV3-1 virus contained the engineered, chimeric PIV3-1 HN and F genes. As designed, the genetic structure of rPIV3-1 was unique in four junction regions when compared with either of its parents, PIV1/Wash64 or rPIV3/JS (boxed in Figure 15A). These regions are the transition points at which the sequence switches from the PIV3 non-coding region to the PIV1 coding region and then from the PIV1 coding region back to the PIV3 non-coding region. Using the primer pair A specific to PIV3 M and L genes, or primer pair B specific to the PIV1 M and the very 3'-end of HN gene, RT-PCR products were generated for virion-derived RNAs from rPIV3-1, rPIV3/JS, and PIV1/Wash64. Control reactions showed that the RT step was required for generation of RT-PCR products, indicating that an RNA template, rather than contaminating DNA, was required to produce the RT-PCR product. An early indication that rPIV3-1

was indeed a chimeric virus came from the finding that only the PIV3-specific primer pair A generated the expected 4.6 kb cDNA product that spans the F and HN genes (Fig. 15B). Thus, while rPIV3-1 virus contains only HPIV1-specific HN glycoprotein (See Table 12), the non-coding regions are specific to PIV3. Conversely, the PIV1 specific primer pair B amplified an appropriately sized product from PIV1 control but not from rPIV3-1. Restriction digestion analysis also demonstrated that rPIV3-1 RT-PCR product had unique restriction patterns different from that of rPIV3/JS and PIV1/Wash64 and appropriate for its predicted sequence.

The nt sequence of the 4.6 kb RT-PCR product of rPIV3-1 was determined in its four regions (Fig. 15 A) and compared with that of rPIV3/JS and PIV1/Wash64 (Fig. 16). The rPIV3-1 sequence was completely in agreement with the cDNA from which it was derived. Examination of the sequence alignment of the Region I-IV for the three RT-PCR products illustrates that rPIV3-1 contains the PIV1 F and HN glycoprotein ORFs with altered start and stop codons and flanked by the 5' and 3' non-coding regions of PIV3. Examples of sequencing ladders spanning the Region III and IV of rPIV3-1 (Fig. 17), compared in parallel with rPIV3/JS or PIV1/Wash64, were evaluated, and this analysis confirmed that rPIV3-1 is a recombinant chimeric virus whose structure is completely in agreement with the cDNA from which it was generated.

Trypsin-dependence and cytopathicity of rPIV3-1 in vitro.

PIV1, like Sendai virus but contrary to PIV3, requires trypsin for cleavage of its F glycoprotein in order to undergo multicycle replication on continuous lines of tissue culture cells (Frank et al. *J. Clin. Microbiol.* 10:32-6 (1979)). In addition, PIV1 is a non-cytopathic virus whereas PIV3 readily produces extensive CPE (Collins et al. In *Fields Virology*, 3rd ed., 1:1205-43 (1996)). rPIV3-1, rPIV3 and PIV1/Wash64 were compared on the basis of these properties. rPIV3-1, like PIV1/Wash64, had a higher HA titer using chicken, rather than guinea pig (Table 13), RBCs. rPIV3-1,

like its PIV1/Wash64 parent, required trypsin for efficient replication in cultures with fluid overlay as well as for efficient plaque formation. rPIV3-1 produced plaques at 32°C, 37°C or 40°C with similar efficiency. It is therefore evident that rPIV3-1 possesses the F glycoprotein of the PIV1 parent virus, and it is not temperature sensitive. On the other hand, rPIV3-1 produced CPE, as indicated by the cell rounding and detaching in the virus infected monolayers, almost to the same extent as its PIV3 parent suggesting that this biological property is a function of its PIV3 genes, which lie outside of the HN and F coding regions. Thus, rPIV3-1 possesses biological properties from both parents which is consistent with the findings above demonstrating that it is a chimeric virus. This exemplary recombinant, chimeric virus within the invention was deposited on May 21, 1998 under the terms of the Budapest Treaty with the American Type Culture Collection (ATCC) of 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A.

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Table 13. Comparison of the HA titer and the infectivity and cytopathicity of parental and chimeric PIVs^a

Virus	HA titer using indicated RBC		Infectious titer ^b (\log_{10} TCID ₅₀ /ml) using CPE or HAD as endpoint				PFU/ml ^c (\log_{10})	
	Chicken	Guinea Pig	CPE		HAD		Trypsin	Trypsin
			-	+	-	+		
PIV1/Wash64	16	8	≤2.5	≤2.5	4.8 ^d	6.3	≤0.7 ^e	5.8
rPIV3-1	64	16	≤2.5	5.8	5.5 ^d	7.8	≤0.7 ^e	7.1
rPIV3/JS	0	8	4.5	7.3	5.0 ^d	7.5	5.0	6.2

^aVirus stocks were grown in LLC-MK2 cells which were infected at an MOI of 0.01 and incubated for 6 days in the presence (PIV1/Wash64, rPIV3-1) or absence (rPIV3/JS) of 0.75 µg/ml trypsin. The resulting virus stocks were assayed by the tests below in the presence or absence of trypsin as indicated.

^bThe TCID₅₀ assay was read at 6 days by direct visualization of CPE or by hemadsorption (HAD).

^cPlaques were visualized by HAD after six days of incubation.

^dThe HAD of PIV3-infected monolayers was grossly apparent whereas that of PIV1 and rPIV3-1 was observable only under the microscope in which single cells with RBC adsorbed were observed.

^eThe lowest level of virus detectable was 10^{0.7}/ml.

Comparison of the level of replication of rPIV3-1 and its parental viruses in LLC-MK2 cells and hamsters.

The multicycle replication of rPIV3, rPIV3-1, and PIV1 Wash/64 viruses was evaluated following inoculation of LLC-MK2 tissue culture cells at a MOI of 0.01 (Fig. 18). It can be seen that the kinetics and magnitude of replication of the three viruses are very similar. This indicates that the substitution of the HN and F genes of PIV1 for those of PIV3 did not attenuate the virus for replication *in vitro*. It was next determined whether rPIV3-1 was attenuated *in vivo*, specifically for replication in the upper and lower respiratory tracts of hamsters (TABLE 14). The observed level of replication of rPIV3-1 was similar to, if not slightly higher than, either parent in the upper and lower respiratory tract of hamsters.

Table 14. Level of replication of parental and chimeric PIVs in the upper and lower respiratory tract of hamsters^a

Virus	Virus titer (mean \log_{10} TCID ₅₀ /gram \pm S.E.) in indicated tissue			
	Nasal Turbinates		Lungs	
	Day 4	Day 5	Day 4	Day 5
PIV1/Wash64	5.2 \pm .24	5.2 \pm .12	5.0 \pm .31	5.0 \pm .38
rPIV3-1	4.9 \pm .23	6.2 \pm .17	5.8 \pm .15	6.0 \pm .09
rPIV3/JS	4.5 \pm .09	5.0 \pm .18	5.1 \pm .26	5.0 \pm .32

^aHamsters were infected intranasally with $10^{5.0}$ TCID₅₀ per animal of the indicated virus, and lungs and nasal turbinates were removed on day 4 or 5 after infection. The titers are means of six animals per day and are expressed as mean \log_{10} TCID₅₀/gram \pm standard error.

In summary, the present Example demonstrates successful recovery of a rPIV3-1 chimeric virus in which the ORFs of the PIV1 HN and F glycoproteins were substituted for those of rPIV3. This chimeric virus replicated like its wild type PIV1 and PIV3 parental viruses *in vitro* and *in vivo*, demonstrating that the substitution of the glycoprotein ORFs did not result in attenuation of rPIV3-1. This successful recovery of a recombinant PIV3 which bears the HN and F glycoproteins of PIV1 is surprising because the two viruses, representing distinct serotypes, are not closely related. In particular, it is remarkable that the chimeric recombinant grows as well as the two parents. Notably, chimeric recombinant viruses possessing a substitution in the glycoprotein gene have also been recovered for vesicular stomatitis virus (VSV) (Lawson et al. Proc. Natl. Acad. Sci. USA 92:4477-81 (1995)). Specifically, the VSV G glycoprotein gene of Indiana serotype was replaced by that from New Jersey serotype which share only 50% amino acid sequence identity. In contrast to rPIV3-1, the chimeric recombinant VSV_{I/NJ} replicates to only 10% the level of recombinant VSV_I or biologically derived, VSV.

In the present Example, the HN and F glycoproteins have 43 and 47% sequence identity, respectively, between PIV1

and PIV3. The transfer of the two glycoproteins together would, of course, obviate glycoprotein-to-glycoprotein incompatibility (Tanabayashi, K. and Compans, R.W. *J. Virol.* 70:6112-18 (1996)). On the other hand, it is generally thought that the glycoproteins interact with the M protein (which is 63% identical between PIV1 and PIV3) through their cytoplasmic (CT) or transmembrane (TM) domains, and that this interaction is important in virion morphogenesis and structure. In this regard, the degree of sequence identity between the HN and F proteins of the two serotypes in the TM and CT domains is low indeed: 30% and 22%, respectively for the TM domain, and 9 and 11% respectively for the CT domain. In light of this low level of sequence relatedness, we have also pursued a parallel strategy of constructing chimeric glycoproteins in which the PIV1 ectodomain of each glycoprotein was fused to the PIV3 TM and CT domains. Regarding possible interaction with the M protein or other internal proteins, it might be that a conserved structure, such as a constellation of charged amino acids, is important rather than a conserved sequence. Alternatively, it might be that interaction of the TM and CT domains of the glycoproteins with internal proteins is not as critical as has been previously thought. It will be possible to examine these factors more closely using the methods and tools provided herein. For example, these factors will be further elucidated by work in progress employing the methods described above to construct a PIV3 virus bearing HN and F of PIV2.

It was expected that rPIV3-1 would require trypsin for efficient replication in tissue culture since this is a property conferred by the PIV1 F glycoprotein, and this was found to be the case. However, it was interesting to observe that rPIV3-1 caused CPE that more closely resembled that of PIV3 parent virus, indicating that a PIV3 gene(s) other than HN or F specifies this phenotype. These roles will also be further elucidated using the methods and tools provided herein to exchange additional gene(s) between the non-cytopathic PIV1 and the cytopathic PIV3.

EXAMPLE XII

Recovery of Live-Attenuated Chimeric Recombinant PIV Encoding
the Internal Proteins of PIV Type 3 and the Surface
Glycoproteins of PIV Type 1

In the present Example, a derivative of rPIV3-1 carrying the three temperature-sensitive and attenuating amino acid coding changes found in the L gene of the live-attenuated cp45 PIV3 candidate vaccine virus, termed rPIV3-1.cp45L, is shown to exhibit a temperature sensitive phenotype with a shut-off temperature of 38°C, similar to that of the recombinant rPIV3cp45L which possesses the same three mutations. rPIV3-1.cp45L is attenuated in the respiratory tract of hamsters to the same extent as rPIV3cp45L. Infection of hamsters with rPIV3-1.cp45L generates a moderate level of hemagglutination-inhibiting antibodies against wt PIV1 and induces complete resistance to challenge with wild type PIV1. This demonstrates that attenuated chimeric PIV according to the invention are capable of inducing a highly effective immune response against PIV1. This disclosure also confirms the above described data demonstrating that the surface glycoproteins of parainfluenza viruses are sufficient to induce a high level of resistance to homologous virus challenge. Unexpectedly, infection with recombinant chimeric virus rPIV3-1.cp45L or rPIV3-1, each bearing the surface glycoprotein genes of PIV1 and the internal genes of PIV3, also induces a moderate level of resistance to replication of PIV3 challenge virus. This indicates that the internal genes of PIV3 can independently induce protective immunity against PIV3 in rodents. Thus, a reverse genetics system for PIV3 as disclosed herein successfully produces live attenuated PIV1 vaccine candidates that are attenuated and protective in accepted model subjects.

viruses and cells.

The wt PIV1 strain used in this study is PIV1/Washington/20993/1964 (PIV1/Wash64) (see, e.g., Murphy et al., Infect. Immun. 12:62, 1975 (incorporated herein by reference in its entirety)). Chimeric rPIV3-1, recovered from chimeric PIV3 cDNA in which the PIV3 F and HN ORFs were

replaced with those of PIV1/Wash64, as described above and in Tao et al., J. Virol. 72:2955, 1998 (incorporated herein by reference in its entirety). These viruses were propagated in LLC-MK2 cells (ATCC CCL 7.1) in Opti-MEM I (Life Technologies, Gaithersburg, MD) with 50 µg/ml gentamicin sulfate, and 0.75 µg/ml trypsin (Catalog No. 3741, Worthington Biochemical Corp., Freehold, NJ). Trypsin is included because the F glycoprotein of PIV1, but not that of PIV3, is dependent on exogenous trypsin for cleavage when grown in cell culture under these conditions. The wt JS strain of human PIV3 virus and its recombinant derivative from cDNA (rPIV3/JS) were propagated as described above and in Durbin et al., Virology 235:323, 1997 (incorporated herein by reference in its entirety). The propagation of cp45, an attenuated derivative of wt PIV3/JS (see above; and Karron et al., J. Infect. Dis. 171:1107, 1995 (incorporated herein by reference in its entirety)), and rPIV3cp45L, a recombinant PIV3 carrying the three ts mutations found in the L gene of cp45, were propagated as described above and in Skiadopoulos et al., J. Virol. 72:1762, 1998 (incorporated herein by reference in its entirety). The modified vaccinia Ankara (MVA) recombinant that expresses the bacteriophage T7 RNA polymerase is described in Virology 210: 202, 1995 (incorporated herein by reference in its entirety).

HEp-2 cells, which are used in transfection, were obtained from ATCC (ATCC CCL 23) and maintained in Opti-MEM I with 2% fetal bovine serum (FBS), 50 µg/ml gentamicin sulfate.

Introduction of L mutations into rPIV3-1 antigenomic cDNA.

The three L mutations of cp45 present in the pTM(L) 942/992/1558 plasmid, described above (see also, Skiadopoulos et al., J. Virol. 72:1762, 1998, were introduced into chimeric cDNA pFLC.2G+.hc (described above; see also, Tao et al., J. Virol. 72:2955, 1998), as a 2.8 kb SphI-NheI fragment (nt 11313 to 14092 in PIV3 antigenomic cDNA) to generate the full-length pFLC.2G+.hc.cp45L bearing the PIV1 F and HN ORFs and the three cp45 L gene mutations (Fig. 19).

The specific mutations present in pTM(L) 942/992/1558 are indicated in the legend to Figure 19.

Transfection. HEp-2 cell monolayers in six-well plates were grown to confluence and transfections were performed as described above (see also, Tao et al., J Virol 72:2955, 1998). Trypsin was added to a final concentration of 0.75 µg/ml on day 3 post transfection prior to harvesting on day 4. Cell culture supernatants were clarified and passaged (referred to as passage 1) onto fresh LLC-MK2 cell monolayers. After overnight adsorption, the medium was replaced with fresh Opti-MEM I with 0.75 µg/ml trypsin. Passage 1 cultures were incubated at 32°C for 4 days, and the virus present in the supernatant was harvested and passaged again under the same conditions (referred to as passage 2). Virus present in the passage 2 harvest was tested for the presence of the PIV1 HN protein by hemagglutination-inhibition (HAI) assay as described above (see also Tao et al., J Virol 72:2955, 1998).

Replication of PIVs in LLC-MK2 at various temperatures.

Plaque enumeration on LLC-MK2 monolayers was performed as described above, with 0.75 µg/ml trypsin added to the agarose overlay in the case of PIV1, rPIV3-1, and rPIV3-1.*cp45L* (see also, Tao et al., J Virol 72:2955, 1998). After incubation at various temperatures for 6 days, the agarose overlay was removed and plaques were identified by hemadsorption (HAD) with guinea pig erythrocytes (RBCs).

Replication of PIVs in the respiratory tract of hamsters.

Groups of five hamsters were inoculated intranasally with 0.1 ml of L15 medium containing 10⁶ plaque forming units (PFU) of rPIV3/JS, rPIV3*cp45L*, *cp45*, PIV1/Wash64, rPIV3-1, or rPIV3-1.*cp45L*. Hamsters were sacrificed on day 4 post-infection, and their lungs and nasal turbinates were harvested and homogenized. Virus present in the tissue samples was titered on LLC-MK2 cell monolayers at 32°C as described above and in Tao et al., J Virol 72:2955, 1998. The titers are expressed as reciprocal mean log₁₀TCID₅₀/gram of tissue for each group.

Immunization and challenge studies in hamsters.

Groups of ten hamsters were immunized intranasally with 10^6 PFU of virus per animal, as described above. Serum was collected for HAI assay prior to infection and on day 33. The level of HAI antibodies present in the sera of each group of 10 hamsters was determined using PIV1/Wash64 and PIV3/JS as antigens, and the HAI titers determined are presented as mean \log_2 (see also, Tao et al., J Virol 72:2955, 1998).

Thirty-five days post-immunization, five hamsters from each group were challenged intranasally with 10^6 PFU of either PIV1/Wash64 or rPIV3/JS. Nasal turbinates and lungs of these challenged hamsters were harvested four days post challenge. Virus titers in tissue samples were determined on LLC-MK2 monolayers as described above and in Tao et al., J Virol 72:2955, 1998, and the titers are presented as mean \log_{10} TCID₅₀/gram of tissue.

RESULTS**Recovery and characterization of the recombinant chimeric virus rPIV3-1.cp45L.**

As noted above, the cDNA clone pFLC.2G+.hc, a full-length antigenomic cDNA of PIV3 in which the ORFs encoding the F and HN glycoproteins have been replaced by those of PIV1, was modified by introduction of three amino acid coding changes (designated 942, 992 and 1558, according to amino acid position in the L protein) identified in the L gene of cp45 and shown to be independent ts and attenuating mutations (Fig. 19; see also, Skiadopoulos et al., J Virol 72:1762, 1998). Each coding change was marked by the co-introduction of contiguous translationally silent nt substitutions that ablate a naturally-occurring restriction site (Fig. 19; Table 8). The final full-length plasmid construct, pFLC.2G+.hc.cp45L (Fig. 19), encodes a PIV3-1 chimeric antigenomic RNA of 15516 nt in length and conforms to the rule of six (see, Durbin et al., Virology 234:74, 1997, incorporated herein by reference in its entirety). The authenticity of pFLC.2G+.hc.cp45L was confirmed by digestion with appropriate restriction enzymes.

The pFLC.2G+.hc.*cp45L* cDNA was transfected into HEp-2 cells together with the PIV3 N, P and L support plasmids and infected with MVA-T7 as described above and in Tao et al., J Virol 72:2955, 1998). Virus recovered after two passages on LLC-MK2 cells, termed rPIV3-1.*cp45L*, was biologically cloned by plaque-to-plaque-to-plaque passage, and amplified virus was analyzed to confirm that it possessed the PIV1 glycoproteins and the three introduced mutations in L. First, the presence of the PIV1 HN protein in rPIV3-1.*cp45L* was confirmed by reactivity with PIV1 specific antibodies in HAI assay as described above and in Tao et al., J Virol 72:2955, 1998). The presence of the chimeric PIV3-1 HN and F genes as well as the introduced L gene mutations in rPIV3-1.*cp45L* genomic RNA was confirmed by restriction enzyme digestion or nucleotide sequence analysis of RT-PCR products generated from virion RNA as described above and in Tao et al., J Virol 72:2955, 1998. These data confirmed that rPIV3-1.*cp45L* is a recombinant chimeric virus bearing the three codon substitutions of the L gene of *cp45*.

rPIV3-1.*cp45L* is temperature sensitive.

The three L gene mutations of *cp45* were shown above to confer the *ts* phenotype when introduced into wt PIV3 (see also, Skiadopoulos et al., J Virol 72:1762, 1998). To evaluate whether their presence in the chimeric virus would have the same effect, the efficiency of plaque formation of rPIV3-1.*cp45L* was determined at various temperatures. As shown in Table 15, the three L mutations indeed conferred the *ts* phenotype to the chimeric virus. The level of temperature sensitivity specified by the *cp45* L mutations in the recombinant viruses rPIV3*cp45L* and rPIV3-1.*cp45L* was equivalent (Table 15), indicating that the effect of the mutations is independent of the PIV3 or PIV1 HN and F glycoproteins. The level of temperature sensitivity of rPIV3*cp45L* and rPIV3-1.*cp45L* was comparable to that of the biologically derived *cp45* virus, despite the fact that the latter virus possesses mutations outside of L (see, Stokes et al., Virus Res 30:43, 1993, incorporated herein by reference in its entirety).

5 **Table 15. The recombinant chimeric rPIV3-1.cp45L candidate vaccine virus is temperature sensitive**

Virus ^a	Virus titer ^b at indicated temperatures (log ₁₀ PFU/ml)					
	32°C	36°C	37°C	38°C	39°C	40°C
rPIV3/JS	7.4	7.2	7.2	7.2	7.2	7.2
PIV1/Wash64 ^c	7.5	7.6	7.5	7.5	7.4	7.2
rPIV3-1	7.5	7.5	7.5	7.2	6.0	6.1
PIV3cp45 ^c	7.4	6.9	6.8	4.7 ^d	<0.7	<0.7
rPIV3cp45L	7.9	7.4	7.7	5.3	1.2	<0.7
rPIV3-1.cp45L	8.1	8.0	8.2	6.1	<0.7	<0.7

10 ^a Virus nomenclature: rPIV3/JS, recombinant wt PIV3 strain JS; PIV1/Wash64, biologically-derived wt PIV1; rPIV3-1, recombinant chimeric PIV3 in which the F and HN ORFs have been replaced with those of PIV1/Wash64; PIV3cp45, biologically-derived cp45 candidate vaccine virus; rPIV3cp45L, recombinant PIV3 containing the three L gene mutations of cp45; rPIV3-1.cp45L, recombinant chimeric rPIV3-1 containing the three L gene mutations of cp45.

15 ^b Virus titers were determined using LLC-MK2 monolayers in 12-well plates. Titers are the average of two assays.

20 ^c Biologically-derived viruses. All others are recombinant viruses.

25 ^d The shut-off temperature, i.e. the lowest restrictive temperature at which a two log₁₀ reduction in virus titer is observed, of each ts virus is indicated in bold.

30 Level of replication of rPIV3-1.cp45L in hamsters.

35 The three L gene mutations of cp45 were shown above to confer attenuation of virus replication in the upper and lower respiratory tract of hamsters when introduced into wt PIV3 (see also, Skiadopoulos et al., *J Virol* 72:1762, 1998). Their effect on the chimeric virus was evaluated by intranasal infection of hamsters, as shown in Table 16. These findings indicate that rPIV3-1.cp45L indeed was attenuated at both sites and, furthermore, that its level of attenuation was comparable to that of rPIV3cp45L. Thus, the ability of the cp45 L mutations to confer attenuation, like temperature sensitivity, is independent of the antigenic specificity of the surface glycoproteins.

Table 16. The recombinant chimeric rPIV3-1.cp45L candidate vaccine virus is attenuated in the respiratory tract of hamsters ^a

5	Virus	Virus titer in indicated tissue (\log_{10} TCID ₅₀ /g±S.E.) ^b	
		Nasal turbinates	Lungs
	rPIV3-1.cp45L	4.6±0.3	1.9±0.4
	rPIV3-1	6.0±0.3	6.3±0.4
10	rPIV3 cp45L	3.0±0.3	<1.2
	rPIV3/JS	5.7±0.3	5.0±0.3

^a Groups of five hamsters were infected intranasally with indicated viruses at a dosage of 10⁶ PFU per hamster. On day 4 post infection, the tissue samples were harvested and assayed for virus.

^b Virus titers are given as Log₁₀TCID₅₀ per gram of tissue.

Infection with rPIV3-1 or rPIV3-1.cp45L, containing the internal proteins of PIV3 and the glycoproteins of PIV1, confers resistance to PIV1 challenge in hamsters.

The chimeric rPIV3-1 virus and its attenuated rPIV3-1.cp45L derivative were evaluated for immunogenicity and protective efficacy in hamsters. As shown in Table 17, infection with either virus induced HAI antibodies against PIV1, but not PIV3, confirming that these chimeric viruses possess the PIV1 HN glycoprotein and are highly immunogenic. The level of HAI antibodies induced by rPIV3-1.cp45L was two-fold less than that by rPIV3-1, which indicates that its attenuation resulted in a modest decrease in immunogenicity. Similarly, rPIV3 and rPIV3cp45L induced HAI antibodies against PIV3, but not PIV1, and the level induced by the attenuated virus was approximately two-fold lower. Despite the restricted replication in hamsters of the recombinant viruses bearing the cp45 L mutations, infection with either rPIV3cp45L or rPIV3-1.cp45L induced complete resistance to replication of challenge virus bearing homologous glycoproteins.

Table 17. The recombinant chimeric rPIV3-1.cp45L candidate vaccine virus induces complete resistance to PIV1 and partial resistance to PIV3 upon challenge in hamsters^a

Virus used for immunization	Origin of glycoproteins	Post-immunization HAI titer (log ₂ Reciprocal+S.E.)		Virus Titer in Indicated Tissue ^c (log ₁₀ TCID ₅₀ /Gram+S.E.)		
				Replication of PIV1 challenge virus		Replication of PIV3 challenge virus
		α-PIV1	α-PIV3	Nasal Turbinates	Lungs	Nasal Turbinates
Control ^b	-	≤1	≤1	5.0±0.3	4.6±0.5	5.5±0.4
rPIV3-1.cp45L	PIV1	6.9±0.5	≤1	≤1.2	1.9±0.6	1.7±0.5
rPIV3-1	PIV1	7.9±0.4	≤1	≤1.2	2.9±0.3	2.6±0.4
rPIV3cp45L	PIV3	≤1	9.3±0.2	4.6±0.2	2.8±0.7	≤1.2
rPIV3/JS	PIV3	≤1	10.3±0.3	4.6±0.4	2.4±0.5	≤1.2
PIV3cp45 ^d	PIV3	≤1	8.9±0.4	4.9±0.2	2.5±0.8	≤1.2

^a Groups of 10 hamsters were immunized intranasally with 10⁶ PFU of indicated viruses. Post-immunization sera were collected on days 33, two days prior to challenge (see ^c).

^b Hamsters in control group were not inoculated.

^c Five weeks after immunization, five hamsters from each group were challenged intranasally with 10⁶ PFU of indicated virus. Tissue samples were harvested 4 days post challenge. Viruses present in tissue samples were titrated on LLC-MK2 monolayers and the data are presented as log₁₀TCID₅₀/gram of tissue ± standard error.

^d Biologically-derived virus.

Infection with rPIV3-1.cp45L also confers resistance to PIV3.

Information on the role of the non-HN or F glycoproteins of PIVs (i.e., the internal proteins) in resistance is limited. The disclosure and use of rPIV3-1 and rPIV3-1.cp45L herein provides an opportunity to examine the role that internal proteins play in resistance to challenge with PIV3, since the only genes shared by immunizing and challenge viruses are the internal protein genes. PIV3 challenge virus replication was significantly restricted in both the upper and lower respiratory tracts by prior infection of hamsters with rPIV3-1 or rPIV3-1.cp45L (Table 17). Thus, these data indicate that the internal proteins of PIV3, like the HN and F proteins, are capable of inducing partial resistance to replication of the challenge PIV3.

Among the findings demonstrated by the immunogenicity and efficacy studies above a particularly unexpected finding was that infection with wt or attenuated rPIV3 induced a 100-fold reduction in the replication of PIV1 challenge virus in the lungs. Thus, infection with one serotype of PIV provided significant protection against a heterologous serotype. This was unexpected in part because previous studies indicated that infection of animals with one type of human PIV did not induce significant heterologous protection against a PIV belonging to a different human serotype, conforming to a general belief that immunity to human PIV infections was largely type-specific (see, eg., Cook et al., Amer. Jour. Hyg. 77:150, 1963; Ray et al., J. Infect. Dis. 162:746, 1990, each incorporated herein by reference in its entirety).

The present Example demonstrates successful exploitation of novel methods and reagents developed for generating PIV3 vaccines to further provide rapid, rational development of live attenuated candidate vaccines for PIV1. A cDNA encoding infectious PIV3 was modified by substitution of the ORFs encoding the PIV1 HN and F protective antigens for their PIV3 counterparts. Subsequently, attenuating mutations, exemplified by three attenuating mutations present in the L

gene of the *cp45* PIV3, were incorporated within this modified chimeric PIV3-PIV1 cDNA. From this cDNA, a recombinant virus was recovered bearing the HN and F genes of PIV1, the internal proteins of PIV3, and the PIV3 *cp45* L gene mutations. This recombinant, rPIV3-1.*cp45L*, was temperature sensitive, highly attenuated in hamsters, and highly efficacious against PIV1 challenge in hamsters. The level of temperature sensitivity, attenuation, and immunogenicity exhibited by rPIV3-1.*cp45L* was comparable to that of *cp45* PIV3, indicating that the phenotypes specified by the set of *cp45* L gene mutations are independent of the HN and F surface glycoproteins. These findings, which represent the first live attenuated PIV1 vaccine candidate generated by reverse genetics, provide a generally successful scheme for developing vaccines against PIV1.

Little information is known concerning the role that internal proteins of parainfluenza viruses play in resistance to reinfection with homologous virus. Infection with vaccinia recombinants expressing N, epitopes within N, or M reportedly induce resistance to replication of challenge virus, but the magnitude of the resistance reported is less than that induced by vaccinia recombinants bearing HN or F glycoproteins (see, e.g., Kast et al., Proc. Natl. Acad. Sci. USA 88:2283, 1991; Sakaguchi et al., J. Gen. Virol. 74:479, 1993; Thomson et al., J. Immunol. 157:822, 1996, each incorporated herein by reference in its entirety). These studies suggested that the internal proteins were making only minor contributions to resistance to reinfection. Therefore, the present disclosure presents unexpected results by showing that prior infection of hamsters with rPIV3-1.*cp45L* or rPIV3-1 induced about 250-to 4000-fold reduction of replication of PIV3 in both the nasal turbinates and lungs. These two chimeric recombinant viruses differ from the PIV3 challenge virus in that they possess the HN and F glycoproteins of PIV1 rather than PIV3, but they share all other genes with the challenge virus. The HN and F glycoproteins of PIV1 share 47% and 43% sequence identity with those of PIV3, respectively. Although it is likely that the shared internal proteins are mediating the observed

resistance, it is also possible that shared protein sequences between PIV1 and PIV3 F and HN glycoproteins are contributing to the observed immunity. For example, there are 5 stretches in HN and 2 stretches in F extending at least 9 amino acid residues in length that are shared between PIV1 and PIV3 and have the potential to act as protective CTL epitopes. It is reasonable to consider that the shared internal proteins are contributing to the restriction of replication of wt PIV3 challenge virus, since this level of cross-immunity has not been seen in previous studies (see, eg., Cook et al., Amer. Jour. Hyg. 77:150, 1963; Ray et al., J. Infect. Dis. 162:746, 1990, incorporated herein by reference in its entirety).

The finding that the internal PIV3 proteins of the rPIV3-1 and rPIV3-1.cp45L chimeras conferred resistance to PIV3 challenge demonstrates that attenuated derivatives of PIV3 can be used as vectors for PIV1 and PIV2 protective antigens. Following the teachings of the invention, immunization with one PIV3-based live-attenuated vaccine virus can restrict the replication of other PIV3-based vaccine viruses administered subsequently, thereby decreasing the immunogenicity of the second virus. Since PIV3, like RSV, induces significant illness in early infancy, a combined RSV-PIV3 vaccine for use in the very young 2- to 4-week old infant is therefore an important aspect of the invention (see, eg., Collins et al., Fields Virology 3rd ed. Philadelphia: Lippincott-Raven Publishers, 1205(1), 1996; Reed et al., J. Infect. Dis. 175:807, 1997, each incorporated herein by reference in its entirety). According to this aspect of the invention, immunization with a PIV1-PIV2 vaccine will be preferably initiated at about 6 months of age, since most PIV1 and PIV2 disease occurs after the age of six months. In the possible circumstance that immunization with rPIV3 cp45 significantly inhibits replication of a chimeric recombinant PIV3-1 vaccine virus with which it shares internal protein genes, successful immunization with a recombinant PIV3-1 vaccine may be compromised. In this event, a trivalent PIV vaccine will be administered simultaneously rather than sequentially, thereby preventing the above noted inhibition.

The disclosure herein that infection with a vaccine or wt PIV3 would induce a 100-fold reduction of pulmonary virus replication of the heterologous wt PIV1 was clearly unexpected, in part because the human PIV viruses are serologically distinct by neutralization assay, and previous studies in hamsters found that prior infection with one type of PIV failed to induce resistance to challenge with a high dose of a different PIV type (see eg., Cook et al., Amer. Jour. Hyg. 77:150, 1963; Ray et al., J. Infect. Dis. 162:746, 1990; Cook et al., Amer. Jour. Hyg. 69:250, 1959). Furthermore, there is little epidemiological data documenting that prior infection with one PIV significantly modifies subsequent infection with a heterotypic PIV.

In summary, the present Example shows that rPIV3 was successfully converted into a vaccine for PIV1 by substituting the ORFs encoding the F and HN glycoproteins and introducing known attenuating mutations into the PIV3 internal genes. Thus, the extensive methods and reagents provided herein can be applied directly and predictably to attenuating the PIV3 backbone of the rPIV3-1 chimeric virus, as well as for generating live-attenuated PIV2 vaccine viruses.

The foregoing disclosure makes it possible to exploit the reagents and methods provided herein to develop a broad assemblage of PIV and related vaccines. In this context, recovery of live, immunogenic chimeras between PIV3 and PIV2 exemplifies powerful new tools for developing a range of recombinant PIV viruses for vaccine use. In conjunction with this work, identification and characterization of the genetic basis for attenuation of naturally occurring PIV mutants, e.g., cp45 and BPIV3 vaccine candidates, following the teachings of the present disclosure also enables development of a large host of recombinant vaccine viruses and subviral particles. In particular, desired mutations present in biologically derived mutant viruses will be readily identified and selected by their introduction, singly and in combination, into a wild type, partially attenuated, or chimeric PIV background, as shown in the Examples above. These findings will expand the menu of exemplary, attenuating mutations

within the invention which can be introduced into PIV clones to calibrate the level of attenuation and immunogenicity in vaccine recombinants. Biologically derived mutations can also be introduced within PIV clones having different types of mutations, e.g., mutations involving alterations, deletions, or substitutions of a gene or gene segment. Within this aspect of the invention, recombinant PIV are provided which have a selected gene deletion, addition, or substitution, such as rPIV having a deletion of the C, D or V ORF(s). Such alternatively mutated clones can be further modified according to the present disclosure by introducing one or more mutations specifying a *ts*, *ca* or *att* phenotype adopted from a biologically derived mutant PIV, as exemplified by the PIV recombinants r942, r992, r1558, r942/992, r992/1558, or r942/1558, and r942/992/1558. In additional aspects of the invention, biologically derived mutations will be combined with *de novo* attenuating mutations not found in nature, as exemplified by attenuating gene deletions, e.g., of the C, D and/or V ORFs. Other types of mutations disclosed herein conferring desired phenotypic characteristics will also be combined with biologically derived, attenuating mutations, similar to the range of combinatorial mutations disclosed for recombinant RSV vaccine strains in United States Patent Application Serial No. 08/892,403, filed July 15, 1997 (incorporated herein by reference). Comparable mutations can be readily introduced, e.g., into a chimeric virus, to achieve a desired levels of attenuation and immunogenicity in a chimeric vaccine strain. In this manner, a large menu of mutations are provided within the invention that are useful to engineer a wide assemblage of live attenuated rPIV vaccines having a desired balance of attenuation and immunogenicity, along with other desired phenotypic characteristics.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.